EXHIBIT B

SEAL

MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT
GENERAL DIRECTORATE OF INDUSTRIAL PRODUCTION
Italian Patent and Trademark Office

****

SEAL OF THE ABOVE MINISTRY

Authentication of copy of documents concerning the Patent application for INDUSTRIAL INVENTION No. RM97A000743

It is hereby certified that the attached copy is the true copy of the original documents filed with the above mentioned patent application whose data are shown in the enclosed filing certificate, furthermore: receipt of reserves dissolution registration No. RMR0091 of 30 Jan. 1998 for the description and drawings (71 pages); request for taking over representation registration No. 803640 of 15 Oct. 1998 filed before the Italian Patent and Trademark Office (3 pages); receipt of the Provincial Office of Industry Commerce and Handicraft in Milan No. Mi-E 000907 for the assignment in favour of Istituto Superiore di Sanità having seat in Rome (8 pages); formal amendment and insertion request registration No. 803745 of 20 Oct. 1998 (131 pages); formal amendment request registration No. 803901 of 30 Nov. 1998 (3 pages).

Rome, date

THE DIRECTOR
OF THE DIVISION

(signature)

SEAL

FORM A
DUTY STAMP

TO THE MINISTRY OF INDUSTRY COMMERCE AND HANDICRAFT
Main Patent Office - ROME
Patent Application for Industrial Invention, filing of reserves,
advanced opening to public inspection

A. Applicant (1) G.S. Physical person
1) Name Ensoli Barbara
Residence Via Festo Avieno 176 – 00136 ROMA code NSLBBR60A63E472K
2) Name G.S.
Residence code

B. APPLICANT'S REPRESENTATIVE BEFORE M.P.O.
Surname, name Maria Vittoria PRIMICERI et al Fiscal code
Name of the corresponding office NOTARBARTOLO & GERVASI S.p.A.
Address Via Savoia 82 city ROME zip code 00198 province RM

C. ELECTED DOMICILE OF THE ADDRESSEE As above
Address city code prov

D. TITLE proposed class, (sec./cl./ucl.) group/subgroup

HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes

ADVANCED OPENING TO PUBLIC INSPECTION yes___ no____
in presence of amendment request: date no. of ref.:

E. NAMED INVENTORS
surname, name surname, name
1) Barbara Ensoli 3)
2) 4)

F. PRIORITY
Country or Exhibition Type of Priority Appln. No. Appln. date Encl(yes/res)

1) NONE

2)

G. CENTRE FOR COLLECTING MICROORGANISMS'CULTURES, denomination

None

H. SPECIAL NOTES
None

ENCLOSED DOCUMENTS

Specimen No.					RESERVES DISSOLUTION
Doc. 1) 1	prov.	no. sheets 24	abstract with main drawing, spec. and claims (compulsory 1 copy)		date No. of ref.
Doc. 2) 1	prov.'	no. sheets 09	(compulsory if cited in description., 1 copy)		
Doc. 3) 1	res.		power of attorney or reference attorney		
Doc. 4) 0	res.		designation of inventor		
Doc. 5) 0	res.		priority document with Italian translation		comparison single priority
Doc. 6) 0	res.		authorisation or assignment deed		
Doc. 7) 0	res.		complete name of the applicant		

8) PAYMENT RECEIPT OF LIT. 565.000.= compulsory
filled in on 01.12.1997

The applicant's signature Dr. Maria Vittoria Primiceri
(signature)

follows yes/no no
We required certified copy of the present deed yes/no yes

PROVINCIAL OFFICE OF INDUSTRY COMMERCE HANDICRAFT OF ROME code 58
FILING CERTIFICATE Application no. RM97A000743 Reg. A
The year 1997 the 1st day of the month of December

The above mentioned applicant(s) has(have) presented to me undersigned the present application consisting of no. 00 additional sheets for the grant of the above patent.

I. DIFFERENT NOTES OF THE RECORDING OFFICER
none

THE DEPOSITER
(signature)

THE RECORDING OFFICER
(signature)
SEAL

FORM A

ABSTRACT OF THE INVENTION TOGETHER WITH MAIN DRAWING, SPECIFICATIONS AND CLAIMS

Application No. RM97A000743 Reg. A
Patent No.

Filing date 01.12.1997
Date of grant

D. TITLE

"HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes"

L. ABSTRACT

The present invention refers to a vaccine, prophylactic or therapeutic, anti-HIV, anti AIDS and against tumours and syndromes associated to HIV infections utilising proteins, peptides and DNA (wild-type or mutant) of HIV Tat, alone or associated to proteins, peptides and DNA of other viral products (Nef, Rev, Gag) or of cytochines having an enhancing action on antiviral immune response.
The invention refers also to the immuniization by means of otology, dendritic cells, mucosal immunization or ex-vivo immunisation of peripheral blood cells expanded through co-stimulation with monoclonal antibodies anti-CD3 and anti-CD28.

M. DRAWING

DESCRIPTION

of the patent application for industrial invention having for title:

"HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes"

in the name of Barbara Ensoli

named inventor: Barbara Ensoli

RM97A000743

*****

The present invention refers to a vaccine, prophylactic or therapeutic, anti-HIV, anti AIDS and against tumours and syndromes associated to HIV infections utilising proteins, peptides and DNA (wild-type or mutant) of HIV Tat, alone or associated to proteins, peptides and DNA of other viral products (Nef, Rev, Gag) or of cytochines having an enhancing action on antiviral immune response.
The invention refers also to the immuniization by means of otology, dendritic cells, mucosal immunization or ex-vivo immunisation of peripheral blood cells expanded through co-stimulation with monoclonal antibodies anti-CD3 and anti-CD28.

Background of invention

AIDS (acquired immunodeficiency syndrome) is caused by HIV virus and characterised by immunodeficiency, tumours, mainly Kaposi sarcoma (KS) and B-cells lymphomas, opportunistic infections and central nervous system alterations. Since AIDS is world-wide spread and has a high mortality, one of the most important public health goals is a prophylactic and/or therapeutical anti-HIV vaccine. Therefore it was mainly utilised as immunogen the viral envelope or sub-units thereof, but with unsatisfactory result due to the extreme variability of the viral envelope. [Wain-Hobson, Curr. Opin. Genet. Dev. 3:878 (1993); Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics, Los Alamos, NH, 1995)]. Therefore it is common opinion that, as an alternative to sterilising immunity, it could be sufficient to have the infection progression stop (therapeutical vaccine). Moreover, immunoprotective responses can be obtained utilising as immunogens, DNA regiones of pathogenic agent [Lu et al., J. Virol. 70:3978 (1996); Boyer et al., Nature Med. 3:526 (1997)]. Owing to the published experimental data, the inventor thinks it necessary to utilise a vaccine produced with the most stable viral products other than env, between those isolated from HIV, inducing an effective both umoral and cellular response, and having a vital function for the virus.
Such products must be experimented in the model of non human primates in that their immune system is more similar to the human one than that of philogenetically more distant animals and in which IDS develops after infection. HIV-1 Tat regulating protein has all such characteristics: it is stable, immunogenic and essential for the early phases of the viral infection. Moreover, Tat has the fundamental role not only in the viral replication and in infection transmission and progression, but also has a starting and progression factor of AIDS associated tumours, for instance KS, which is the most frequently AIDS associated tumour and of other syndromes and syntoms developing after HIV infection.
Tat is a protein of 86-102 amino acids, depending on the viral strain, codified by two exons. Tat is produced soon after the infection, localises itself in the nucleus and transactivates the expression of the viral genes interacting with the target sequence "Tat-responsive element" (TAR) of LTR [Chang et al., J. Biomed. Sci. 2:189 (1995)]. Tat could also have a role in the HIV virulence through other action mechanisms in the viral cycle [Huang et al., EMBO J. 13:2886 (1994); Neuvet et al., J. Virol. 70:5572 (1996); Harrich et al., EMBO J. 16:6 (1997); Li et al., Proc. Natl. Acad. Sci. USA 94:8116 (1997)]. The product of the first exon (amino acids 1-72) is preserved in different viral extracts [Myers et al., Human retroviruses and AIDS. Theoretical Biology and Biophysics. Los Alamos. (1995)] and is sufficient for the transactivation of HIV-1 [Chang et al., J. Biomed. Sci. 2:189 (1995)]. It contains 4 domains. The acidic domain (amino acids 1-21) is important for the Tat interaction with cellular proteins; the cysteine reach region (amino acids 22-37) represents the transactivation domain. This region is the more preserved one in the

primary extracts [Meyerhans et al., Cell 58:901 (1989)]. The substitution of cysteine 22 with a glycine cancels the Tat capacity of transactivating the HIV-LTR [Yang et al., J. Virol. 70:4576 (1996)]; the core region (amino acids 38-48) is also preserved and is important for the activation and substitution of lysine 41 with a threonine, inactivates the transactivating activity of Tat on HIV LTR [Kashanchi et al., J. Virol. 70:5503 (1996)]; the basic domain (amino acids 49-57), reach in arginine and lysine, is necessary for the nuclear localisation and specifically bonds its target RNA (TAR) [Chang et al., J. Biomed. Sci. 2:189 (1995)]. Moreover, the basic region is responsible for the bonding of extracellular Tat to the eparine and to the proteoglycan-eparansulphates (HSPG) [Chang et al., AIDS 11:1421 (1997)]. Mutation in the basic region cancels such interactions. The carboxyl terminal portion of Tat is not necessary for the LTR transactivation, but contains a argynine-glycine-aspartic acid sequence (RGD), usually present in the extracellular matrix proteins (ECM), responsible for the Tat bonding to integrinic receptors $\alpha_5\beta_1$ e $\alpha_v\beta_3$ and for the Tat effects on AIDS associated tumours and on immune, vascular and nervous system [Barillari et al., Proc. Natl. Acad. Sci. USA 149:3727 (1993); Ensoli et al., Nature 371:674 (1994); Zauli et al., Blood 80:3036 (1996); Chang et. al., J. Biomed. Sci. 2: 189 (1995)]. During the acute infection of T-cells with HIV-1 or after the transfection of tat gene in COS-1 cells, the Tat protein is released in absence of cellular death in the extracellular environment [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Chang et al., J. Biomed. Sci. 2:189 (1995)]. The Tat release from infected cells occurs also in vivo since Tat is present in infected subjects serum [Westendrop et al., Nature 375:497 (1995)] and in the AIDS-KS lesions [Ensoli et al., Nature 371:674 (1994)]. After the release, part of the protein remains in a soluble form, while the other one links itself to the HSPG of ECM. Tat linked to the HSPG can be recovered in an eparine soluble form. The bond with eparine is due to the Tat basic region, prevents the effects of the extracellular Tat and protects the protein fro the oxidation, to the point that it permitted us the purification of high biological activity Tat [Chang et al., AIDS 11:1421 (1997)]. Extracellular Tat can be absorbed by the cells, can migrate in the nucleus and can transactivate the viral genome expression [Frankel et al., Cell 55:1189 (1988); Mann et al., EMBO J. 10:1733 (1991); Marcuzzi et al., J. Virol. 66:4228 (1992); Ensoli et al., J. Virol. 67:277 (1993)]. The absorption of That occurs by endocytosys mediated by the RGD bond to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [Barillari et al., Proc. Natl. Acad. Sci. USA 90:7941 (1993) Ensoli et al., Nature 371:674 (1994)] and/or through endocytosys mediated by the basic region bonding to HSPG.

Tat can activate the viral replication and the infection diffusion also through indirect mechanisms involving the modulation of the expression of cellular genes which play a key role in the cells survival control, and of inflammatory cytochines action on viral replication [Chang et al., J. Biomed. Sci. 2:189 (1995)].

Beyond its importance in the viral replication, Tat plays a very important role in the AIDS pathogenesis. That is able to modulate the survival and proliferation of infected and non-infected cells by causing activation or repression of cytochines, such as IL-2 [Puri et al., AIDS Res. 11:31 (1995); Westendorp et al., J. Virol. 68:4177 (1994); Chirmule et al., J. Virol. 69:492 (1995)], or of genes having a key role in the cellular cycle [Sharma et al., Biochem. Biophys. Res. Co. 208:704 (1995); Zauli et al., Blood 86:3823 (1995); Li et al., Science 268:229 (1995); Westendorp et al., Nature 375:497 (1995); Gibellini et al., Blood 89:1654 (1997)]. The anti- or pro-apoptotic effects of Tat depend on a number of factors

such as cellular type, the fact that Tat is expressed from the cell or added to the cell and from its concentration [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Zauli et al., J.Immunol. 157:2216 (1996)].

Tat is the factor responsible for the enhanced frequency and aggressiveness of KS in HIV-1 infected subjects [Ensoli et al., AIDS Updates, Eds. V. De Vita, Jr., Hellman S., Rosenberg S.A., Lippincott J.B., Philadelphia; 7: 1 (1994); Corallini et al., Cancer Res. 53: 1 (1993)]. KS is a vascular originating tumour and the most frequent neoplasia in virus infected subjects. Tat induces the KS cells and the endothelial cells activated by inflammatory cytochines (IC) to migrate, to express collagenasys IV, to invade ECM and to proliferate, such processes being necessary for the angiogenesys and the tumoral invasion. [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994); Albini et al., Proc. Natl. Acad. Sci. USA 92:4838 (1995); Fiorelli et al., J. Clin. Invest. 95:1723 (1995)]. Such Tat effects are induced by IC, in that they stimulate the Tat receptors expression, l'$\alpha_5\beta_1$ and $\alpha_v\beta_3$ [Barillari et al., Proc. Natl. Acad. Sci. 90: 7941 (1993)]. Tat mimes the ECM proteins effect, such as fibronectine and vitronectine and the region containing RGD and the basic region are necessary for the effects of the extracellular Tat on KS cells, on angiogenesys and on progression of KS. The evidence of extracellular Tat presence and of its in vivo bond to the Tat receptors in the AIDS-KS lesions [Ensoli et al., Nature 345: 84, 1994] supports the idea that Tat is involved in the onset and the manteinment of the KA associated to AIDS. Moreover gene tat for transgenic rats develop KS or other phenotype tumours depending on the expression level of the transgene [Vogel et al., Nature 335: 601 (1988); Corallini et al., Cancer Res, 53: 5569 (1993)].

It was suggested that Tat plays o role in the iperproliferative phenomena and in the pathogenesis of the B lymphomas, frequently observed in sieropositive subjects and in tat transgenic rats [Vellutini et al., AIDS Res. Hum. Retrov. 11:21 (1995)], through mechanisms involving the enhancement of bcl-2 and cytochines expression [Puri et al., Cancer Res., 52:3787 (1992)]. Other evidences confirm a probable role of Tat in oncogenesis [Kim et al., Oncogene 7: 1525 (1992)].

Tat can also activate the expression of viral promoters, such as those of the herpesviruses and of other viruses which reactivate themselves in AIDS subjects, promoting the onset and progression of opportunistic infections [Chang et al., J. Biomed. Sci. 2:189 (1995)].

Tat seams also able to exert neurotoxic effects both direct (through the basic region and the RGD, and indirect through induction of inflammatory cytochines having a toxic effect on the neurones of the central nervous system or on the ematoenchephalic barrier [(Chang et al., J. Biomed. Sci. 2:189 (1995)]. In adult and paediatric subjects the central nervous system alterations can lead to dementia and to learning and behavioural alterations.

As far as the Tat immune response is concerned a number a studies suggest that anti-Tat antibodies play a protective role and can control the illness evolution in vivo [Reiss et al., J. Med Virol. 30:163 (1990); Rodman et al., Proc. Natl. Acad. Sci. USA 90:7719 (1993); Rodman et al., J. Exp. Med. 175:1247 (1992); Re et al., J. Acquir. Immun. Defic. Syndr. 10:408 (1996)]. Moreover, in vitro, anti-Tat antibodies not only suppress the absorption, the transcellular activation of Tat and the infection [Ensoli et al., J. Virol. 67:277 (1993); Re et al., J. Acq. Immun. Def. Synd. 10:408 (1995)], but also in inhibit the AIDS-KS cells proliferation and Tat induced migration and the formation of KS-like lesions in rats [Ensoli

et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994)]. Moreover, our preliminary results show that anti-Tat antibodies are absent in AIDS-KS subjects, suggesting that such subjects do not have antibody activity blocking the extracellular Tat and, hence, that this protein can act undisturbed.

The development of an anti-Tat cell-mediated response in the initial phase of the infection is important for the control of the infection itself [Voss et al., Virology 208:770 (1995); Rinaldo et al., AIDS Res. Hum. Retrov. 11:481 (1995); Harrer et al., AIDS Res. Hum. Retrov. 12:585 (1996)] and there exists an inverse correlation between the specific anti-Tat CTL presence and illness progression [van Baal et., J. Gen. Virol. 78:1913 (1997)]. Such results were obtained in studies on macaques inoculated with SIVmac [Lu et al., J. Virol. 70:3978 (1996); Venet et al., J. Immunol. 148:2899 (1992)]. Moreover, recent works on rats of different species in which Tat was inoculated both as plasmide and as protein did show that it is possible to induce both a umoral and cellular response towards the protein [Hinkula et al., J. Virol. 71:5528 (1997)] though it was observed a variability between rat species and that such results were not been reproduced with the same immunogens in a work on non-human primates [Quesada-Rolander et al., ABS 6-S1, 2nd European Conference on Experimental AIDS Research, Stockholm, Sweden, May 31-June 3 (1997)]. The lack of reproducibility in the vaccine experimentation in rats with respect to the one on non-human primates is frequent and possibly due to the different immune system in the two animal species which can bring to different immune responses with the same immunogen, as demonstrated for HIV with the virus Env protein. All that requires that the immunogens proposed for vaccine experimentation for men must tested on non-human primates and not only in inferior species. The inventor believes that other viral proteins, or parts thereof, could be associated with Tat and could enhance an immune response specific to HIV and could be of benefit also in the vaccination against onset of tumours and of other pathologies and symptoms associated to HIV infection. Such products are the HIV Nef, Rev and Gag proteins.

Nef is another viral regulating protein important for the development of the illness [(Allan et al., Science 230:813 (1985); Franchini et al., Virology 155:593 (1986); Guy et al., Nature 330:266 (1987)] early produced after the infection and released in the extracellular environment. In the system SIVmac/macaque the presence of Nef is correlated with high doses of viral amount and with progression towards AIDS [(Kestler et al., Science 248:1109 (1991)]. Nef is more variable than Tat [(Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics. Los Alamos, NH (1995)]. Nef is an immunogenic protein [(Gobert et al., Virology 176:458 (1990); Choppin et al., J. Immunol. 147:569 (1991); Culman et al., J. Immunol. 146:1560 (1991); Tahtinen et al., Virology 187:156 (1992)], and it is able to induce CTL [(Bourgault et al., J. Virol. 66:75 (1992); Couillin et al., J. Exp. Med. 180:1129 (1994)]. In particular, it was pointed out an immunodominant region in the Nef central region (region 73-144) which is recognised in the largest part of patients with anti-Nef CTL.

Rev is a viral regulating protein early produced during infection [Gait et al., Trends Biochem. Sci. 18:255 (1993); Parslow, Human Retroviruses, Ed. B.R. Cullen, IRL press, Oxford, England, p. 101 (1993)] and released in the extracellular environment. Rev is essential for the HIV replication and for the illness progression, and is codified by two exons, partially superimposed to the ones codifying for That. Rev is a nuclear protein [Felber et al., Proc. Natl. Acad. Sci. 86:1495 (1989)] necessary for the expression of the

RNAs viral messengers for the tardy proteins [Malim et al., Nature 338:254 (1989)]. Rev is a highly preserved protein in the various viral extracts of HIV-1 [Meyers et al., Human Retroviruses and AIDS: A compilation and analysis of nucleic acid and amino acid sequences, Los Alamos Laboratory, Los Alamos, NM p.1 (1993)] and it is immunogenic. In fact it induces the production of specific antibodies directed against the two functional domains of the protein [Pilkington et al., Mol. Immunol. 33:439 (1996)] during the natural infection in man [Reiss et al., AIDS Res. Hum. Retrov. 5:621 (1989)] and after inoculation in rats [Hinkula et al., J. Virol. 71:5528 (1997)]. The lowering of anti-Rev antibodies seral levels seems to be correlated with the progression towards AIDS [Reiss et al., AIDS Res. Hum. Retrov. 5:621 (1989)]. Rev can induce CTL both in men and in monkeys [van Baalen et al., J. Gen. Virol. 78:1913 (1997); Venet et al., J. Immunol. 148:2899 (1992)] and it was reported that a specific anti-Rev CTL response, early during the infection is inversely correlated with the illness progression [van Baalen et al., J. Gen. Virol. 78:1913 (1997); Venet et al., J. Immunol. 148:2899 (1992)].

Another viral target is the gag gene, which is tardily expressed during infection and codifies for a group of highly immunogenic structural capsidic proteins [Bruisten et al., J. Infect. Dis. 166:620 (1992); Sipsas et al., J. Clin. Invest. 99:752 (1997)]. The anti-Gag antibody titres maintain themselves high and stable during the asymptomatic phase of the infection, and reach very low levels when the infection goes on to clear AIDS, in combination with the CD4+ lymphocites drop and the finding of the virus in the blood [Baur et al., J. Infect. Dis. 165:419 (1992); Koup et al., J. Virol. 68:4650 (1994)]. Gag protein induce CTL activity early during infection, both in men and in primates [Mcfarland et al., J. Inf. Dis. 170:766 (1994); Yasutomi et al., J. Virol. 70:678 (1996)], and their presence is significantly related with the control of initial viremia and with the illness progress [Klein et al., J. Exp. Med. 181:1365 (1995); Aryoshi et al., AIDS 9:555 (1995); Rinaldo et al., J. Virol., 69:5838 (1995); Yang et al., J. Virol. 70:5799 (1996); Lubaki et al., J. Infect. Dis. 175:1360 (1997)]. Moreover, proteins p17 and p24 contain immunodominant epitopes which are maintained in different extracts of HIV-1 and HIV-2 and recognised by CTL [Littaua et al., J. Virol. 65:40 (1991); Buseyne et al., J. Virol. 67:694 (1993); Nietfield et al., J. Immunol. 154:2189 (1995); van Baalen et al., J. Gen. Virol., 77:1659 (1996); Nixon et al., Nature 336:484 (1988)].

The inventor believes that cytochines or parts thereof, such asIL-12 and IL-15, or other immunomodulant cytochines or in any case enhancing the immunogenic effect, can be utilised as adjuvant in the anti-Tat vaccination. IL-12 is a strong immunoregulatory cytochine produced by cells having the antigen such as B and dendritic cells [Per review see Trinchieri, Curr. Opin. Hematol. 4:59 (1997)]. IL-12 is early produced after HIV infection and has a pro-inflammatory action inducing NK cells and T lymphocytes to produce IFNγ which makes the phagocytes activated and promotes the Th1 lymphocytes induction. IL-12 plays a fundamental role in rising the resistance to a number of infections caused by bacteria, fungi, viruses and shows a high antitumoral activity. It is believed that the immunodepression inducing viruses such as HIV and measles virus, act also through IL-12 production depressing mechanisms. [Grosjean et al, J. Exp. Med. 186: 801 (1997); Fugier-Vivier et al, J. Exp. Med. 186: 813 (1997) Schnorr et al, Proc. Natl. Acad. Sci. USA. 94: 5326 (1997)].

IL-15 is a pleiotropic cytochine expressed by non-lymphoid tissues, by activated monocytes/macrophages and by dendritic cells[Quinn et al., Biochem. Biophys. Res

Commun. 239:6 (1997); Jonuleit et al., J. Immunol. 158:2610 (1997)]. IL-15 plays an important role in regulating the NK activity, in the lymphocytes T proliferation and in the CTL activity [Jullien et al., J. Immunol. 158:800 (1997); Carson et al., J. Clin. Invest. 99:937 (1997)]. IL-15 induces the expression of CTL against HIV antigens, and in IL-2 absence and T CD4+ functional lymphocytes [Kanai et al., J. Immunol. 157:3681 (1996); Agostini et al., Blood 90:1115 (1997)]. Moreover, IL-15 as IL-2, induces the onset of lymphocytes having cytotoxic activity ("lymphokine-activated killer", LAK) and stimulates the IFNγ production in PBMCs of seropositive patients [Lucey et al., Clin. Diagn. Lab. Immunol. 4:43 (1997)]. IL-15 activated the monocytes to produce chemokines, playing a controlling role in the onset of inflammatory processes [Badolato et al., Blood 90:2804 (1997)].

Within the different systems aimed at the generation of effective antiviral and antitumoral vaccines the inventor thinks that utilisation of dendritic cells could be a key element in the induction of the Tat immune response. This is due to the fact that those are the most efficient cells in presenting the antigen and the sole able to stimulate intact lymphocytes, adjuvants being absent [Steinman R.M., Exp. Hematol. 24: 859 (1996)]. The use of dendritic cells replaces the function of a number of adjuvants consisting in the induction of a non specific immunitary response(natural immunity) which in turns generates a potent primary specific response in presence of the antigen.

Since the transmission of HIV infection primarily occurs at mucosal level (genital and rettal in the adult, oral in the new-born), the inventor thinks that the induction of protective immunity at mucosal level is a primary important goal, many works did recently show the possibility to induce local and systemic immunisation. Particularly the nasal and oral route did show to be the most efficient in inducing an effective mucosal immunitary response, even in distant sites, such as genital mucosas [Rosenthal et al., Seminars in Immunology 9:303 (1997); O'Hagan et al., Novel Delivery Systems for Oral Vaccines, Eds. O'Hagan, D.T. CRC Press Boca Raton, FL, p. 176 (1994)].

Recent woks did show that the co-stimulation of CD4+ lymphocytes with paramagnetic beads coated with anti-CD3 e anti-CD28 monoclonal antibodies determines the logarithmic and polyclonal expansion of the lymphocytes coming from HIV-infected subjects [Levine et al., Science. 272: 1939-1943 (1996)] without activating the replication and propagation of the virus. Such antiviral activity is a consequence of the negative modulation of the expression of CCR5 , co-receptor of HIV-1 monocytotropic strains [Carrol et al, Science. 276: 273-276, (1997)] and, in a lesser extent, to the high levels of chemokines induced by co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies [Riley et al, J.Immunol. 158: 5545-5553, (1997)]. The inventor thinks that the possibility to expand autologous lymphocytes from HIV infected subjects in absence of viral replication/propagation, permits to obtain an effective ex vivo immunisation, described in the examples, which can be highly helpful in producing an anti-Tat vaccine.

The inventor thinks that such observations suggest that the immunisation with Tat, alone or in combination with other viral products or immunomodulant cytokines, or parts thereof, could stop the viral replication in subjects exposed after vaccination and in already infected subjects, maintaining the infection in an abortive phase, which therefore could be more easier controlled by the immune system. Therefore the inventor thinks that a Tat based vaccine should be able to induce an immunitary response, both umoral and cellular, sufficient to stop or reduce the replication or the transmission of the virus and therefore to

protect from the infection, from the illness and from the onset of the tumours and of the other syndromes and symptoms associated with AIDS. It is therefore possible to use the anti-Tat vaccine both as prevention as therapy. In fact a umoral response against Tat could neutralise the effects of extracellular Tat reducing and limiting the infection while the cell-induced response against Tat and other viral proteins, enclosed in the vaccine formulation, should destroy the virus replicant cells and therefore to control the infection and to permit to the immune system of the host to develop a complete immune response towards the viral components in absence irreversible damages due to the viral replication.

Claims

The present invention refers to:

1. a prophylactic and therapeutic proteic or peptidic vaccine to be used in men, to be used against AIDS, tumours and syndromes and symptoms associated to HIV infection, comprising recombinant proteins of wild-type Tat and mutants thereof (Seq. 1-5), expressed and purified as described, or of its wild-type or mutant peptides (Pep. 1-7), to be administrated alone or combined with the universal T-helper epitope of tetanic toxoid or other T-helper peptides;
2. a vaccine as described in the above, combined with recombinant proteins or with Nef, Rev or Gag peptides of HIV, or administrated in form of Tat/Nef, Tat/Rev, Tat/Gag fusion proteins or part thereof;
3. a vaccine as described in the above, in combination with recombinant proteins of immunomodulating cytokines such as IL-12, IL-15 or other molecules or parts thereof, enhancing the antiviral immune response, or administrated as Tat/IL12, Tat/IL-15 or Tat/other molecules fusion proteins, or parts thereof, enhancing the antiviral immune response;
4. a prophylactic and therapeutic vaccine to be used in men, against AIDS, tumours and syndromes or symptoms associated with HIV infection, comprising vectors which codify Tat wild- type or relevant mutants (Seq. 1-5), or parts thereof, inserted in the PCVO plasmidic vector other vectors;
5. a DNA vaccine as described at point 4, in combination with DNA molecules inserted in the pCV0 plasmidic vector or other vectors, of HIV rev, nef e gag genes or parts thereof, or administrated as plasmids co-expressing tat/rev, tat/nef, tat/gag or parts thereof;
6. a DNA vaccine as described in the above, in combination DNA molecules based on pCV0 plasmid or other vectors, of the genes for the immunomodulating cytokines IL-12, IL-15 or other immunomodulating genes, or parts thereof, enhancing the antiviral immune response, or administrated as plasmid co-expressing tat/IL-12, tat/IL-15 or tat/other molecules, or parts thereof, able to enhance the antiviral immune response;
7. a proteic, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the immunisation with autologue dendritic cells by means of ex vivo treatment;
8. a proteic, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the mucosal immunisation (nasal, oral, vaginal or rettal);
9. a protein, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the immunisation ex vivo of infected subjects peripheral blood cells expanded though co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies and re-infused in the host;
10. a proteic, peptidic and/or DNA anti-Tat vaccine as described, combined with viral

replication inhibitors.

Detailed description of the invention

The present invention relates to a prophylactic and therapeutic vaccine against the HIV infection, the progression towards AIDS, the developments of tumours and of other syndromes and symptoms in subjects infected by the virus, utilising recombinant, proteic, peptidic and DNA- molecules of HIV-Tat. More particularly the invention refers to a vaccine based on HIV-1 Tat as immunogen, inoculated as DNA and/or recombinant protein or of peptides, alone or in combination with other genes or viral genetic products (Nef, Rev, Gag) or parts thereof, or in combination with various immunomodulating cytokines (IL-12, IL-15) or with codifying gene for an immunomodulating cytokine or part thereof. Tat, Nef, Rev, Gag and the immunomodulating cytokines are administrated both as a mixture of recombinant proteins, of peptides or of fusion proteins (Tat/Nef, Tat/Rev, Tat/Gag, Tat/IL-12, Tat/IL-15) and as plasmidic DNA. It is also described an immunisation method utilising autologous dendritic cells treated ex vivo with recombinant Tat protein, or peptides thereof, alone or with a mixture of recombinant proteins or peptides (Tat, Nef, Rev, Gag) or with the Tat protein and one or more immunomodulating cytokines, or parts thereof, or transduced with eucariotic vectors containing Tat alone or with codifying viral genes for Nef, Gag or Rev, or Tat and the codifying gene for an immunomodulating cytokine or part thereof.

It is also described the induction use of an immune response at the mucosal level. Tat or its peptides, alone or in combination with viral proteins and/or citokines is inoculated also at mucosal level to enhance and induce the local immunitary response. The HIV- Tat protein or sub-.units thereof will also be utilised for the *ex vivo* immunisation of CD4+ and CD8+ lymphocytes insulated from the peripheral blood of infected subjects. Later the Tat antigen specific cells will be expanded in vitro through co-stimulation with monoclonal antibodies directed against CD3 and CD28 and re-infused In is also described the use of Tat mutants, identified in the examples, to be utilised as immunogens, as an alternative to Tat wild type. The Tat mutants are i) in the cysteine region (cys22) and ii) in the core region (lys41), iii) the deleted mutant in the RGD sequence; iv) the double mutant in lysine 41 and in RGD. Alternatively to the use of Tat mutants or Tat peptides (wild type or mutated as the protein) in case of therapeutic vaccination, along with the immunogen, inhibitors of the viral replication will be utilised.

The present invention will be now described by means of its illustrative and not limitative specific examples, in which reference will be made to the enclosed figures.

Brief description of the figures

FIG. 1. CAT test to determine the capability of purified Tat-cys22 (Tat22) protein to compete with the transactivating activity of Tat wild type protein. H3T1 cells, containing the HIV-1 LTR-CAT "reporter" vector integrated in its genome [Sodroski et al., Science 227:171, (1985)], in which the gene for the chloramphenicol acetyl-transferase enzyme (CAT) is placed under transcriptional control of the LTR promoter of HIV-1, have been incubated in presence of Tat wild type protein (100 ng) alone or in combination with a molar excess of Tat-cys22 protein (1 μg). The transactivating activity of Tat on the HIV-1 LTR and the capability of the Tat-cys22 protein to compete with Tat wild type have been determined after 48 h, by subjecting amounts of the cytoplasmic extracts (corresponding to 200 μg of protein) to the assay for determination of the activity of the cAT enzyme, as described [Ensoli et al., J. Virol. 67:277 (1993)]. The percentages (%) in acetylation of

^{14}C-chloramphenicol are indicated.

FIG. 2 . Umoral response of anti-Tat specific IgG type in monkeys vaccinated with the Tat protein, determined by immuno-enzymatic assays (ELISA). (A) shows the results obtained on two monkeys inoculated with 10 or 100 μg of Tat recombinant protein re-suspended in 250 μl autologous serum and 250 μl RIBI, subcutaneously in a site; (B) shows the results for the non-inoculated control monkey. Monkeys were inoculated at 0 time and after 2, 5, 10, 15, 22 and 27 weeks. The presence of the anti-Tat antibody response in the vaccinated animals plasma was evaluated by ELISA assays prepared and characterised by us. The Tat protein was adsorbed at 96 well plates in PVC (100 ng/well in 200 μl carbonate buffer 0.05 M pH 9.6) for 12 h at 4°C. After 3 washings with PBS-A 1x containing Tween 20 (0.05%), sera were added (in duplicate) diluted 1:50 in 200 μl carbonate buffer, it was incubated at 37°C for 90', the wells were washed with PBS-A 1x/Tween 0.05% and the presence of the immunocomplexes was highlighted by adding 100 μl of secondary antibody (diluted 1:1000 in PBS-A 1x/Tween 0.1%/BSA 1%) conjugated with horseradish peroxidase, for 90' at room temperature. After 5 washings of the wells, 100 μl of substrate of the peroxidase (ABTS 1 mM, Amersham) were added for 30-45' at room temperature and then a spectrophotometric reading was carried out at 405 nm. Each ELISA essay included a polyclonal serum included anti-Tat rabbit polyclonal serum (positive control) diluted 1:200 to 1:6400, and the monkeys sera taken at 0 time (negative control) diluted 1:50. The cut off value was considered as (± S.D.) mean of the readings of the sera of all monkeys, taken at 0 time, obtained in all the experiments. The results shown by the histogram for each sample correspond to the mean optical densities at 405 nm of the readings of the two wells, subtracted of the cut off value ± S.D (ΔOD405). >2,7: The read value were out of scale.

FIG. 3. Titration of the plasma of monkeys inoculated with 100 and 10 μg recombinant Tat protein, described in fig. 2. The results in ordinate are represented as the inverse of the highest dilution of the serum which at 405 nm had a reading higher than the cut off value.

FIG. 4. Analysis of the specific anti-Tat umoral response of IgM type in monkeys inoculated with Tat and determined by ELISA. essay. Three monkeys (M1-3) subcutaneously inoculated with 10 μg recombinant Tat protein re-suspended in 250 μl autologous serum and 250 μl RIBI and 3 monkeys (M4-6) subcutaneously inoculated with 10 μg recombinant Tat re-suspended 250 μl autologous serum and 250 μl Alum; 2 control monkeys subcutaneously inoculated with RIBI (250 μl and 250 μl autologous serum) (M7) and with Alum (250 μl and 250 μl autologous serum) (M8). The monkeys were inoculated at 0 time and after 2, 6, 11 and 15 weeks. The ELISA essays were performed and the cut off values were determined as described in fig. 2. In this case the sera of the animals were tested (in duplicate) at dilution 1:100 and monkey anti-IgM goat serum conjugated with horseradish peroxidase was used as secondary antibody, diluted at 1:1000.

FIG. 5. Analysis of the umoral response of anti-Tat specific IgG type in monkeys inoculated with Tat tested by ELISA. Three monkeys (M1-3) inoculated with 10 μg of recombinant Tat re-suspended in 250 μl autologous serum and 250 μl RIBI and 3 monkeys (M4-6) inoculated with 10 μg recombinant Tat resuspended in 250 μl autologous serum and 250 μl Alum; two control monkeys inoculated with RIBI (250 μl and 250 μl autologous serum) (M7) or with Alum (250 μl and 250 μl autologous serum) (M8). The monkeys were inoculated at 0 time and after 2, 6, 11 and 15 weeks. The ELISA essays

were carried out and the cut off values were determined as described in fig. 2. >2,7: the value was out of scale.

FIG. 6. Titration of the sera of the monkeys inoculated with recombinant Tat (10 μg) in presence of RIBI adjuvant (M1-3) or Alum (M4-6). The results are shown for each serum as the inverse of the highest serum dilution which at 405 nm gave a reading higher the cut off value.

FIG. 7. Analysis of the response of delayed ipersensitivity to Tat by performing skin test. Tat protein (5, 1 and 0.2 μg), re-suspended in 150 μl PBS containing 0.1% BSA and the buffer in which Tat is resuspended were intradermally inoculated in a shaved area on the animal back. The area was photographed at 0 time and after 24, 48 and 72 hours. The control monkeys were inoculated only with buffer. In the figure it is shown an example corresponding to the 15th week for the M2 monkey at 48 h after the inoculation of the immunogens. It is evident a strong positive reaction to Tat.

FIG. 8. Kinetic of the PBMC proliferative response of *Macaca fascicularis* to the co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies on paramagnetic beads (anti-CD3/28 beads). The PBMC insulated from peripheral blood were depleted of the CD8 positive sub-population by immunomagnetic methods. Afterwards, part of anti CD8-depleted lymphocytes was stimulated with PHA and IL-2 (40U/ml) starting from the 3 day; the remaining part was made to adhere on the beads bearing the anti-CD3/28 antibodies, thus obtaining a CD8-depleted and CD3/28 positive lymphocytes population. At this fraction IL-2 (40U/ml) was added starting from the 10 day of culture. The cells were counted and this viability was determined each 2-3 days. The ratio beads:cells was maintained constant. The number of cells during time is reported.

FIG. 9. Antiviral effect of co-stimulation with anti-CD3/28 beads on PBMC of *Macaca fascicularis*. The CD8-depleted and CD8-deplete lymphocytes CD3+/CD28+, obtained with the methods described in fig. 8 from 4 monkeys, were stimulated as described in example 5. The two fractions were infected *in vitro* at the 0 day with 0.1 M.O.I. of SIVmac251/63M. The stimulation was performed with PHA and IL-2 added from day 3, and with the anti-CD3/28 beads without addition of exogenous IL-2. The viral production was evaluated by determining the p27 levels (ng/ml) on the supernatants of the culture at day 6 and 12 from infection as described in example 5.

Example 1. Expression, purification and characterisation of the Tat protein (IIIB insulated), mutants of the Tat protein and wild type peptides and mutants of Tat.

Many difficulties have been encountered in the past to purify and maintain the biological activity of the Tat protein owing to the easiness of its oxidation, aggregation and loss of activity This is due to the high amount of cysteine, which form intra- and inter-molecular bonds thus modifying the conformation of the native protein [Viscidi et al., Science 246:1606 (1989); Ensoli et al., J. Virol. 67:277 (1993)]. The cDNA or the tat gene (Seq. 1, example 2), which has been cloned in the pL-syn vector, furnished by Dr. J. F. DeLamarter and B. Allet (Glaxo Institute for Molecular Biology S.A., Ginevra, Svizzera), has been used for the expression of the protein in E.Coli.

A first method which we used and which lead to obtain an active protein, was based on successive steps of high pressure liquid chromatography liquid and ion-exchange chromatography [Bohan et al., Gene Expr. 2:391 (1992); Ensoli et al., J. Virol. 67:277 (1993)]. The thus obtained protein is pure at more than 95% and it is active [Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994)], however a good

reproducibility was not obtained from batch to batch, owing to the protein oxidation, which is the main problem in commercial Tat preparations. Owing to our observations that Tat basic region has a strong affinity for eparine and that eparine prevents its oxidation, we used the affinity chromatography with heparin and defined a new Tat purification protocol, as described by Chang et al., [AIDS 11: 1421 (1997)]. Cells (10 gr. in weight) of *E.coli* expressing Tat were sonicated in 40 ml of lysis buffer (disodium phosphate 20mM, pH 7.8; glycerol at 2.5%; PMSF 0.2 mM; DTT 5 mM; mannitol 50 mM; ascorbic acid 10 mM; NaCl 500 mM) by using an Ultrasonic Liquid Processor (Model XL2020, Heat System Inc) with three discharges, each of 20 sec.. The lysate was centrifuged at 12,000 g for 30 min. and the supernatant was incubated for one hour at room temperature with 2 ml of resin heparin sepharose, pre-washed with the lysis buffer. The resin was charged on a glass column and washed with the lysis buffer till the protein was no more detected in the washing medium. The bonded material legato was eluted with lysis buffer containing 2M NaCl and the eluate was collected in fractions of 1 ml. The homogeneity of the eluted protein was analysed by gel electrophoresis (SDS-PAGE). The purified protein was stored lyophilised at -70°C and resuspended in buffer degassed before use.

The biological activity of purified Tat protein, according to the above described protocol, was evaluated with an assay of "rescue" of the viral infection in HLM-1 cells, derived from HeLa-CD4+ cells containing defective proviruses in the tat gene, obtained and described by Sadaie et al. [New Biol. 2:479 (1990)]. The "rescue" assay for the viral infection, described by Ensoli et al. [J. Virol. 67:277 (1993)], consisted in complementing the lack of Tat expression in HLM-1 cells (2×10^5) by means of the addition of exogenous Tat protein (2 µg/ml) and evaluating the viral replication by means of the determination, with an ELISA kit, of commercial "antigen capture", of the concentration of p24 antigen released in the culture medium 48 after the addition of the exogenous Tat protein. The results of the "rescue" expressing, described by Chang et al. [AIDS 11: 1421 (1997)], demonstrate that the Tat protein, purified with this method was active and that with this method the purification was better, easier and less expensive both for purity and for biological activity with respect to the previously described method [Ensoli et al., Nature 345:84 (1990), J. Virol. 67:277 (1993), Nature 371:674 (1994)].

Different preparations of recombinant Tat, purified as described in the above were inoculated in presence of Freund adjuvant in mice and rabbits, according to standard protocols [(Antibodies - A laboratory manual, Eds. Harlow E., Lane D., Cold Spring Harbor Laboratory (1988)]. The results of the antibody response induced by the immunisation are shown in Table 1.

TABLE 1.

Analysis of the specific anti-Tat antibody response in sera of mice and rabbits immunised with the recombinant Tat protein[a].

anti-Tat antibody	OD-ELISA/Tat			Western blot
	1:500	1:1000	1:2000	
rabbit	0.651	0.400	0.175	+
rat	0.502	0.240	0.150	+

[a] *The recombinant Tat protein produced in E.coli was utilised to immunise mice and rabbits according to standard immunisation protocols [(Antibodies - A laboratory manual, Eds. Harlow E., Lane D., Cold Spring Harbor Laboratory (1988)]. The sera of the immunised animals were analysed by ELISA assay for the presence of anti-Tat antibodies*

by using three serum dilutions (1:500 at 1:2000). The results are the mean of the readings at 405 nm of two rabbits and three mice. Moreover the sera were tested by Western blot for the recombinant Tat protein (100 ng) migrated in gel SDS-polyacrilamide.

The results of Table 1 demonstrate that the recombinant Tat prepared by us was able to induce an antibody response in both animal species, as tested with ELISA and Western blot, by utilising the recombinant Tat protein as antigen. Such antibodies were able to inhibit the internalisation and the biological activities of Tat [Ensoli et al., Nature 345:84 (1990), J. Virol. 67:277 (1993), Nature 371:674 (1994)]. The pL-syn vector and the purification protocol of Tat protein are used to express and purify the mutants of Tat described in Example 2. The biological activity of the mutated and purified Tat proteins is measured by "rescue" tests for the viral infection in HLM-1 cells, assays of cellular proliferation KS cells and in vivo in mice, as described in the above for the wild-type Tat protein. Moreover, the mutated Tat proteins are tested in presence of wild-type Tat (at serial concentrations) to verify the negative transdominant effect on the viral replication in "rescue" tests in HLM-1 cells. The pL-syn vector and the purification protocol are used to express and purify fusion proteins of this type: Tat (wild type or mutants thereof)/IL-12 or Tat (wild type or mutants thereof)/IL-15 or parts of the same or Tat (wild type or mutants thereof)/other molecules (or parts thereof) able to enhance the immune response to Tat alone or associated with other viral products. Fusion recombinant molecules are bilt utilising the sequences and the primers described in examples 2 and 3. As an alternative, as immunogens synthetic peptides are utilised corresponding to regions of Tat or other viral products or of cytokines to be used in combination with Tat. The peptide sequences of Tat are:

Pep. 1. MEPVDPRLEPWKHPGSQPKT
Pep. 2. ACTNCYCKKCCFHCQVCFIT
Pep. 3. QVCFITKALGISYGRK
Pep. 4. SYGRKKRRQRRRPPQ
Pep. 5. RPPQGSQTHQVSLSKQ
Pep. 6. HQVSLSKQPTSQSRGD
Pep. 7. PTSQSRGDPTGPKE

The Tat mutant peptides will contain the same amino acid substitutions of mutated Tat proteins described in Example 2. The peptides will be utilised in combination with the peptide representing the universal T-helper epitope of the tetanic toxoid or of other T-helper peptides [Lanzavecchia, Science 260: 937 (1993)].

Example 2. Construction and characterisation of tat gene mutants

We produced 19 mutants in different Tat regions by means of site specific mutagenesis or of deletion. The sequence of each mutated DNA was controlled by means of sequencing. The tat gene mutated cDNAs were cloned in the PstI site of the pCV0 vector, described in Example 3. Each mutant was co-transfected, as described by Ensoli et al. [J. Virol. 67:277 (1993)], in COS-1 cells, or in the Jurkat T lymphocytes line, with the HIV-1 LTR-CAT plasmide, in which the Cat reporter gene is placed under the transcriptional control of the HIV-1 LTR. The results of those experiments, not published are reported in Table 2.

TABLE 2.

Effects of Tat mutants on the HIV-1 LTR-CAT transactivation and block effect (negative transdominant) on the Tat wild-type activity

MUTANTS	Transactivating activity [a] Mean (fold)	(min-max values)	Transdominant activity[b] (% inhibition) Mean
CYS 22	0.09	(.021-.22)	21
THR 23	0.36	(.16-1)	
THR 23A	0.30	(.16-.78)	
ASN 24	0.34	(.34-.82)	
ASN 24A	0.42	(.45-.95)	
TYR 26	0.14	(.08-.19)	
LYS 28/29	0.52	(.19-1.04)	
CYS 30	0.30	(.045-.65)	
CYS 31	0.60	(.27-1.09)	
PHE 32	0.31	(.077-.097)	
LYS 33	0.04	(.0027-.068)	46
GLU 35	0.31	(.19-.43)	
PHE 38	0.05	(.043-.057)	98
LYS 41	0.04	(.025-.061)	97
TYR 47	0.58	(.31-.8)	
57 A	0.35	(.26-.44)	
TAT-RGD	0.94	(.73-1.15)	
TAT-KGE	1.11	(.67-1.49)	
TAT wild-type	1	1	

a The results are given as activation increments relevant to CAT activity values induced by the wild-type Tat (Fold=1). b The results are expressed in inhibition percent (%) of the wild-type Tat activity.

From the results presented in Table 2 it can be observed that for the majority of the mutants the transactivating effect of the HIV-1 LTR was very reduced or absent, with the exception of the deletion mutant of the RGD sequence, which had an activity similar to the activity of the wild-type Tat. We selected the 4 mutants (cys22, lys33, phe38, lys41) having the lowest (almost zero) transactivating activity and we determined the negative transdominating effect on the transactivating activity of wild-type Tat. To this end COS-1cells were co-transfected with each vector containing a Tat mutant and the pCV-Tat vector (in a moral ratio of 10:1) in the presence of the HIV-1 LTR-CAT vector. as can be seen in Table 2, the lys41 and tyr47 mutants inhibited almost completely the Tat activity, while the lys33 and cys22 mutant partially inhibited the Tat activity. However, the cys22 recombinant protein (described in following Example 3) was able to compete with the wild-type Tat protein in transactivating the HIV-1 LTR-CAT (fig. 1). A mutant in the cysteine region (cys22), one in the core region (lys41), one in the second deleted exon of Tat of the RGD (RGDΔ) sequence and a double mutant containing the mutation in lys41 and the deletion of the RGD sequence (lys41-RGDΔ) were selected.

The sequence of the tat insert and of the mutants selected for the vaccination is reported hereinafter. A series of tat gene mutants is described prepared trough 1) substitution of a base to obtain an amino acidic substitution and 2) deletion of a base sequence to obtain a deletion of the correspondent amino acids. The bases substitutions and deletions were obtained by site direct mutagenesis. The sequences of the wild-type tat gene and of the tat gene mutants, hereinafter reported, were inserted in the pCV0 plasmidic vector as described in the above.

With Seq. 1 it is intended the HIV-1 tat gene sequence, BH-10 clone and of its derived protein. With Seq. 2 it is intended the cys22 mutant sequence (and of its derived protein), represented by a substitution of Timine (T) nucleotide in position 66 starting from the 5' end with the Guanine (G) nucleotide. This is a substitution in the derived amino acidic sequence of one Cysteine (C in one letter code) in position 22 at the amino-terminal end, with a Glycine (G in one letter code). With Seq. 3 it is intended the lys41 mutant sequence (and of its derived protein), represented by a substitution of the Timine (T) nucleotide in position 123 from the 5' end with the Cytosine (C) nucleotide. This is a substitution in the derived amino acidic sequence of a Lysine (K in one letter code) in position 41 from the amino-terminal end, with a Threonine (T in one letter code). With Seq. 4 it is intended a sequence of the RGD mutant (and of its derived protein), represented by the deletion of the nucleotide sequence CGAGGGGAC, from nucleotide 232 to nucleotide 240, starting from the 5' end of the wild-type tat gene. In the derived amino acidic sequence those is a deletion of the amino acids Arginine-Glycine-Aspartic acid (RGD in one letter code) in the positions 78-80 from the amino-terminal end. With Seq. 5 it is intended a sequence of the double lys41-RGDΔ mutant (and of its derived protein), originated by the combination of the above described mutants.

Wild-type tat nucleotide sequence (Seq. 1)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAA
AACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCA
TAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGCCCACCTCCCAAT
CCCGAGGGGACCCGACAGGCCCGAAGGAATAG 3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Cys22 mutant nucleotide sequence (Seq. 2)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAA
AACTGCGGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCAT
AACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAC
CTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGCCCACCTCCCAATC
CCGAGGGGACCCGACAGGCCCGAAGGAATAG3

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTAGTNCYCKKCCFHCQVCFITKA
LGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-CaOH

Lys41 nucleotide sequence (Seq. 3)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAA
AACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCA
TAACAAACGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGCCCACCTCCCAAT
CCCGAGGGGACCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

RGDΔ mutant nucleotide sequence (Seq. 4)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAA

AACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCA
TAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGCCCACCTCCCAAT
CCCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Lys41-RGDΔ mutant nucleotide sequence (Seq. 5)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAA
AACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCA
TAACAAACGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGCCCACCTCCCAAT
CCCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Example 3. Construction and characterisation of the DNA immunogens.

The DNA molecules for the inoculation of animals are built in the pCV0 plasmide vector of 6.4 kb [Arya et al., Science 229:69 (1985)]. This plasmide comprises two replication origins of SV40, the major late promoter of the adenovirus (AdMLP), and the splicing sequences of the adenovirus and of the mice immunoglobulines genes, the cDNA of mice dihydrofolate-reductase gene (dhfr) and the SV40 polyadenilation signal. The site for the restriction enzyme PstI is located at 3' of the AdMLP, and represents the site in which the exogenous gene of interest is cloned. The tat gene cDNA (261 couples of bases) (Seq. 1, example 2) of HIV was derived from the BH10 strain [Ratner et al., Nature 313:277 (1985)] and codifies for a protein of 86 amino acids. The pCV-Tat vector [Arya et al., Science 229:69 (1985)] was obtained by cloning the tat gene cDNA in the pCV0 PstI site, the cDNA gene being thus under transcriptional control of the AdMLP. The choice of this vector is based on that the AdMLP induced a higher expression and release of Tat, with respect to other eucariotic promoters, such as, for instance, the immediate early region promoter of the cytomegalovirus (CMV) as demonstrated by Ensoli et al. [J. Virol. 67:277 (1993)], and reported in Table 3.

TABLE 3.

Expression, subcellular localisation, release and activity of Tat in COS-1 cells transfected with pCV-Tat and CMV-Tat[a].

Vectors	Tat expression			Tat[b] content			Tat activity	
Positive Cells	Nucleus[c] (%)	Cytoplasm[c]		Total	Intracell (%)	Extracell (%)	Intracell[d] (fold)	Extracell.[e] (cpm)
pCV-Tat	5-10	++	++	25	63.5	36.5	50	2,478
CMV-Tat	3-5	++	+	14.6	92.2	7.8	72	2,254
Control	0	-	-	0	0	0	1	1,400

[a]COS-1 cells (5 x 10^6) were transfected by electroporation with 30 μg of pCV-Tat, CMV-Tat or a control DNA. 48 hours after transfection, the Tat expression was evaluated though immunoistochemistry with anti-Tat monoclonal antibodies (given values are the mean of percentage value of positive cells) and through localisation of nuclear and cytoplasmatic Tat. The presence of intra- and extra-cellular Tat was analysed through

immunoprecipitation on the cellular extracts (500 µl) and in the culture media (4 ml) and subsequent densitometric lecture (Gelscan XL; Pharmacia) of the precipitated Tat bands. The activity of intracellular Tat was measured on cellular extracts of COS-1 cells cotransfected with Tat expressing vectors, or the control vector and the LTR-CAT HIV-1 plasmide; the extracellular Tat activity on the AIDS-KS cells proliferation induction (determined by tritiate-timidine incorporation assay) was measured in the culture medium (diluted 1:2 and 1:4) of the cells transfected with plasmides expressing Tat or the control plasmide. The results correspond to the mean of five independent experiments.

[b]*Densitometric analysis of the immunoprecipitated Tat protein band. Values are expressed in an arbitrary scale, the total detected minimum value (intra- and extracellular Tat) being 10.*

[c]*-, negative; +, 50% of Tat-positive cells; ++, 50-100% of Tat-positive cells.*

[d]*CAT activity after 20 minutes incubation with respect to the control vector, the activation value of which is considered 1.*

[e]*The AIDS-KS cells growth was measured through a tritiated timidine incorporation assay (standard deviation, DS: 12%). The medium of the cells transfected with control DNA had a tritiated timidine incorporation of 1,400 cpm (DS: 11.5%). The culture medium derived from lymphocytes containing HTLV-II virus (positive control) had a tritiated timidine incorporation of 2,400 cpm (DS: 10%).*

From the results of Table 3 it can be observed that in the pCV-Tat transfected cells, compared with the CMV-Tat transfected cells, the Tat-positive cells percentage and the total Tat content are higher, the amount of released Tat is much higher and is related to the total and cytoplasmatic content of Tat, and the biological activity of the extracellular Tat on the AIDS-KS cells growth is in consequence higher. Such results show that the pCV-Tat vector codifies for a biologically active protein, induces high expression levels of tat gene cDNA and can release from the cells much higher Tat amounts than CMV-Tat vector.

The pCV0 vector is utilised also for the expression of HIV-1 nef, rev and gag genes and of the genes codifying for cytokines IL-12 and IL-15. The cDNAs of nef genes (618 couples of bases, NL43 strain) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995)] and rev (348 couples of bases, strain NL43) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995)], the gag gene (1500 couples of bases, strain NL43) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995], or the cDNAs of IL-12 genes [Wolf et al., J. Immunol. 146:3074 (1991)] o IL-15 [Grabstein et al., Science 264:965 (1994)] are amplified with the polymerase chain reaction technique (PCR) by using specific primers complementary to the first 15 nucleotides of region 5' (primer forward) (Seq. P1, P3, P5, P7, P9) or to the last 15 nucleotides of region 3' of the gene (primer reverse) (Seq. P2, P4, P6, P8, P10). Moreover, each primer, both forward and reverse, comprises the sequence for the restriction enzyme PstI, thus being able to consent the clonation of the amplified in the pCV0 vector. After the clonation, the inserted genes sequence is controlled by means of DNA sequencing. The pCV0 vector is used also for the Tat co-expression with other viral genes of HIV-1 (rev, nef or gag) or with the genes of IL-12 o IL-15 cytokines. To this end the cDNA of HIV-1 tat gene of 261 couples of bases (Seq. 1, example 2) is amplified

through PCR with a primer forward including the sequence for the PstI restriction enzyme (Seq. P11) and a primer reverse complementary to the last 15 nucleotides of tat gene (Seq. P12). The viral genes (nef, rev o gag) or the genes of the IL-12 or IL-15 cytokines are amplified with a primer forward which includes also a sequence of 15 bases complementary to the tat gene 3' region, permitting the gene being in frame with the tat gene (Seq. P13, P14, P15, P16, P17), and a primer reverse including the sequence for the PstI restriction enzyme (Seq. P2, P4, P6, P8, P10). Afterwards, a third PCR reaction is performed in which the DNA template is represented by the amplified of the tat gene and of the gene of interest, the primer forward is represented by the primer utilised in amplifying tat (Seq. P11) and the primer reverse by the one utilised in amplifying the gene of interest (Seq. P2, P4, P6, P8, P10). The amplified tat/gene of interest is purified with agarose gel, digested with PstI and cloned in pCV0. After clonation the sequence of inserted genes in controlled by means of DNA sequencing, while the protein expression is determined by means of transfection as described in the above (Ensoli et al, J. Virol. 67; 2771993).

The sequences of the above mentioned primers are:

Seq. P1. Primer forward Rev: 5'ATGGCAGGAAGAAGC3'
Seq. P2. Primer reverse Rev: 5'CTATTCTTTAGTTCC3'
Seq. P3. Primer forward Nef: 5'ATGGGTGGCAAGTGG3'
Seq. P4. Primer reverse Nef: 5'TCAGCAGTCCTTGTA3'
Seq. P5. Primer forward Gag: 5'ATGGGTGCGAGAGCG3'
Seq. P6. Primer reverse Gag: 5'TTATTGTGACGAGGG3'
Seq. P7. Primer forward IL-12: 5'ATGTGGCCCCCTGGG3'
Seq. P8. Primer reverse IL-12: 5'TTAGGAAGCATTCAG3'
Seq. P9. Primer forward IL-15: 5'ATGAGAATTTCGAAA3'
Seq. P10. Primer reverse IL-15: 5'TCAAGAAGTGTTGAT3'
Seq. P11. Primer forward Tat: 5'ATGGAGCCAGTAGAT3'
Seq. P12. Primer reverse Tat: 5'CTATTCCTTCGGGCC3'
Seq. P13. Primer forward Tat/Rev: 5'GGCCCGAAGGAAATGGCA GGAAGAAGC3'
Seq. P14. Primer forward Tat/Nef: 5' GGCCCGAAGGAAATGGGT GGCAAGTGG3'
Seq. P15. Primer forward Tat/Gag: 5' GGCCCGAAGGAAATGGGTGCG AGAGCG3'
Seq. P16. Primer forward Tat/IL-12: 5' GGCCCGAAGGAAATGTGGC CCCCTGGG3'
Seq. P17. Primer forward Tat/IL-15: 5' GGCCCGAAGGAAATGAGAAT TTCGAAA3'

Example 4. Inoculation in healthy *Macaca fascicularis* of a proteic anti-Tat vaccine: evaluation of safety, tolerability, specific immune response.

The tolerability, the safety and the ability to induce a specific immune response (umoral and cellular) of the recombinant Tat protein produced with the above described method and purified with heparin affinity columns were evaluated in experimental model of non human primates of the cynomolgus monkeys (*Macaca fascicularis*). To activate an ample immune response with a proteic vaccine we used aluminum hydroxide (Alum) which was tested in many models and is the only one approved for experiments in men. Among the

dimiculate adjuvants we used RIBI (of the emulsifying agents group and composed by monophosphorilic A lipid, dimiculate trealose and skeleton of the bacterial wall of the Calmette-Guerin bacillus) [Audibert et al., Immunol. Today 14:281 (1993); Morein et al., AIDS Res. Hum. Retrov. S10:S109 (1994)].

In the first pilot experiment we inoculated 3 monkeys according to the following scheme: Recombinant Tat protein (100 μg), resuspended in 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a site. 2). Recombinant Tat protein (10 μg), resuspended in 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a site. 3) Non inoculated control monkey. In days -42 and -35 prior to the first vaccination 10 ml of blood were taken from monkeys to determine the base parameters. Samples of sera and plasma were frozen at -20° or -80°C and utilised lately to resuspend the proteic inoculum. Monkeys 1 and 2 were inoculated at 0 time and after 2, 5, 10, 15, 22 and 27 weeks. In the same days of the immunogen inoculation 10 ml samples of blood were taken to perform the laboratory tests (chemio-clinical analysis, electrolytes, leukocytes, platelets and haemoglobin counts), the evaluation of immunologic parameters, such as the presence of specific immunoglobulines (IgM, IgG, IgA) the Th1 (IL-2, IFNγ) and Th2 (IL-4, IL-10) type cytokines levels, IL-15, the chemokins production (Rantes, MIP-1 and MIP-1), the lymphocyte phenotype (CD4, CD8,CD3, CD14, CD20, CD25, CD56 e HLA-DR, CD45RA and CD45RO), the proliferative response to Tat, the CTL and NK activity presence, and the CD8+ (CAF) cells mediated antiviral activity presence. Moreover, to evaluate the in vivo presence of a cell-mediated response, all the monkeys were vaccinated and the control ones were subjected to skin-test for Tat. This pilot experiment includes a further inoculation of all the monkeys at week 32 and a last inoculation with Tat protein resuspended in Iscom (immune stimulating complex). Iscom is an adjuvant composed by saponine Quil A, cholesterol and phospholipides and able to increase the umoral and cellular response [Morein et al., AIDS Res. Hum. Retrov. 10:S109 (1994); Lövgren et al., Vaccine 14:753 (1996)]. The protective effect of vaccination will be measured after the challenge of monkeys, vaccinated and control, performed at week 41 from the beginning of the immunisation, intravenously inoculating 10 MID_{50} (50% monkey infectious doses) of simian-human immunodeficiency virus (SHIV) developed and titred in *Macaca fascicularis* and containing the tat gene and/or the nef and rev gene of HIV [Shibata et al., J. Virol.65:314 (1991); Li et al., J. AIDS 5:639 (1992); Sakuragi et al., J. Gen. Virol. 73:2983 (1992); Li et al., J. AIDS 5:639 (1992); Igarashi et al., AIDS Res. Hum. Retrov. 10:1021 (1994); Luciw et al., Proc. Natl. Acad. Sci. 92:7490 (1995); Reinmann et al., J. Virol. 31: 98 (1996)]. The post-challenge monitoring (every other week starting from the challenge day for the first month, every 4 weeks for the subsequent 3 months, and every 8 weeks up to 6 months from the challenge) will comprise also the analysis of viral parameters such as determination of the p27 plasmatic values and the viral amount in plasma and in cells. The results of this experiment relating to the first 27 weeks from the beginning of the protocol are as follows. In the vaccinated and control monkeys were not observed significant alterations of physico-chemical, haematological and behavioural parameters. No inflammation or vascular neoformation traces were observed in the inoculation sites. Those results show that the Tat protein was well tolerated by the animals and was non toxic in the administrated doses, utilising the selected inoculation routes. In monkeys 1 and 2 specific anti-Tat IgG antibodies were present starting from the 5th week after the first inoculation. At week 27 anti-Tat IgG were detectable at dilution 1:3200 and

1:6400 in the plasma of monkeys 1 and 2 respectively. The results are reported in figures 2 and 3. The results available at the moment show the onset of a proliferative response to Tat at week 22 (Table 4) in monkeys inoculated with Tat, the response being higher in monkey 2 which received at each inoculation 10 μg of recombinant Tat protein.

TABLE 4

Proliferative response to Tat at week 22 post-immunisation[a]

Monkey N_i	Stimulus	Stimulation index
1	PHA	13,95
	TT	4,68
	Tat	2,45
2	PHA	11,57
	TT	3,77
	Tat	3,04
3	PHA	19,9
	TT	6,23
	Tat	1,38

[a]*Peripheral blood lymphocytes insulated through Ficoll gradient and inoculated at 2x10^5 cells for well in triplicate in 96 well plates; were growth in RPMI 1640 containing 10% of fetal calf serum and stimulated with Tat (5 μg/ml). PHA (4 μg/ml), or tetanic toxoid (TT) (10 μg/ml), against which all the monkeys were vaccinated. Control samples were incubated only with the culture medium. The cellular proliferation increase was determined at fay 5 with tritiated timidine incorporation assay as described [Ensoli et al., IV International Conference on AIDS, Stockholm, 1:241 (1988); Cafaro et al., AIDS Res. Hum. Retrov. 7:204 (1991)]. The results are reported as stimulation index (which was calculated according to the following formula: sample cpm mean/control cpm mean. Values higher than 2) are considered positive. Monkeys 1 and 2 were inoculated with recombinant Tat protein (100 μg or 10 μg, respectively) resuspended n 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a sito. Monkey 3 is a non inoculated control monkey.*

The results of this pilot experiment show that the recombinant Tat protein, produced and purified according to the protocol described by us, was non toxic at subcutaneously inoculated doses of 100 and 10 μg and was able to induce a specific both umoral and cell-mediated immune response. The anti-Tat specific immune response was higher in monkey 2, immunised with 10 μg of recombinant protein. Moreover, also the RIBI adjuvant was apparently non toxic in animals.

In a subsequently started experiment we evaluated the effect of the immunisation induced by Tat in combination with RIBI or with Alum. The monkeys were subcutaneously inoculated in a single site according the following route. Monkeys 1-3 : 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of RIBI. Monkeys 4-6: 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of Alum. Monkey 7: RIBI 250 μl and 250 μl of autologous serum. Monkey 8: Alum 250 μl and 250 μl of autologous serum. From all the monkeys 10 ml of blood were taken at day -9, before the first vaccination, in orther to carry out the analysis described in the first pilot experiment and to have the base parameters of each animal. The monkeys

were inoculated at 0 time and after 2, 6, 11 and 15 weeks. In the same days of the immunogen inoculation 10 ml of blood were taken to carry out the tests described in the first pilot experiment. Moreover, urine samples and vaginal tampons were taken on which the presence of specific secretory IgA will be determined. This pilot experiment involves two boosters at weeks 21 and 26 for monkeys 1-6 with the proteic immunogen and adjuvants and for monkeys 7 and 8 only with adjuvants. Finally, it will be performed a last booster at week 31 with the Tat protein Tat resuspended in Iscom. The protective effect of the vaccination will be therefore determined after the challenge of the vaccinated and control monkeys, which will be performed at week 40 from the beginning of the vaccination, by intravenously inoculating 10 MID_{50} of SHIV. The post-challenge monitoring will be carried out as described for the first pilot experiment.

The results of the second pilot experiment, available at present and relating to week 15 from the beginning of the vaccination, are as follows. In the animals there are not observed significant alterations of physico-chemical, haematological and behavioural parameters. The monkeys did not present inflammation or neo-vascularisation signs in the inoculation sites. It has been demonstrated the presence of a specific antibody response (IgM, IgG). At week 15 the anti-Tat antibodies titres (IgG) reached high values which varied from 1:12.800 to titres higher than 1:50.000 (figures 4-6). Moreover, the neutralising activity for Tat in the monkeys sera was tested by inhibition assays of the rescue of the viral infection in HLM-1 cells treated with the exogenous Tat protein, as described in the above [Ensoli et al., J. Virol. (1993)]. Such tests demonstrate that the monkeys M1-6 sera, diluted 1:2, obtained at week 15 from the first inoculum, were able to block the viral replication induced by exogenous Tat, measured by determining the p24 antigen in the supernatant of the cells, while the serum of the same monkeys at 0 time (pre-immune) or the negative controls did not show activities blocked towards Tat (Table 5).

TABLE 5

Neutralising activity of anti-Tat antibodies on the rescue of the viral infection induced by extracellular Tat.

Samples	HIV-1p24 (pg/ml)
Tat	61.88
Tat + Pre-immune serum M1	46.80
Tat + Pre-immune serum M2	22.96
Tat + Pre-immune serum M3	96.98
Tat + Pre-immune serum M4	126.68
Tat + Pre-immune serum M5	27.03
Tat + Pre-immune serum M6	78.61
Tat + Immune serum M1	4.77
Tat + Immune serum M2	4.88
Tat + Immune serum M3	Neg.
Tat + Immune serum M4	Neg.
Tat + Immune serum M5	7.88
Tat + Immune serum M6	4.55
Pool M1-6 (pre-immune control)	Neg.
Pool M1-6 (immune control)	Neg.
PBS + 0.1% BSA	Neg.

The neutralising activity of the anti-Tat antibodies was determined in HLM-1 cells (HeLa cells containing an integrated copy of the defective for tat gene HIV-1 provirus). HLM-1 were plated at a concentration of 6 x 10^5/plate in 24 wells plates and incubated at

37°C for 16 h. The cells were twice washed with PBS containing bovine serum albumin (BSA) at 0.1%, and incubated for 48 h on culture medium (0.3 ml) to which recombinant Tat protein had been added (30 ng/ml) both alone or in presence of a same volume of monkeys sera (diluted 1:2) taken at 0 time (pre-immune sera) or at week 15 (immune sera). The controls were the cells only treated with a pool of the pre-immune, immune sera, or with PBS containing BSA at 0.1% (PBS + 0.1% BSA) but without Tat. Each sample was double tested. Afterwards, the presence of the cells released virus was tested on the cells supernatants by testing the p24 antigen values, by utilising a p24 antigen capture - Elisa (Nen Dupont) commercial kit. The results are expressed as values of p24 (pg/ml) present in the media and correspond to the mean of the values obtained from two wells for each sample.

At week 11 it was also evident a proliferative response, specific for Tat in monkeys 2 and 3 inoculated with Tat protein and RIBI, and monkey 4 inoculated with Tat protein and Alum, which increased at week 15 (Table 6).

TABLE 6.

Proliferative response to Tat[a]

		Weeks from the beginning of the immunisation			
Monkey N°	Stimulus	0	6	11	15
1	PHA	16,96	22,83	10,50	15,27
	TT	11,69	2,16	1,96	3,01
	Tat	1,12	1,94	1,55	0,52
2	PHA	31,27	29,37	25,75	21,28
	TT	1,12	2,16	1,8	0,57
	Tat	1,08	2,05	3,65	6,22
3	PHA	22,42	39,15	7,89	16,88
	TT	11,43	1,72	0,95	1,71
	Tat	1,65	1,44	2,69	18,82
4	PHA	3,88	13,85	20,77	15,22
	TT	2,85	3,90	4,49	9,07
	Tat	1,29	1,88	3,01	3,24
5	PHA	6,50	11,39	5,74	16,74
	TT	2,31	3,22	1,07	4,84
	Tat	1,80	1,02	0,66	1,76
6	PHA	11,96	7,01	17,94	2,77
	TT	4,14	5,01	1,71	0,13
	Tat	1,37	2,47	1,06	0,11
7	PHA	21,65	25,20	20,30	37,93
	TT	0,97	1,30	0,80	0,88
	Tat	1,78	1,12	0,68	0,73
8	PHA	26,51	21,44	67,09	16,38
	TT	1,20	2,03	10,78	0,20
	Tat	1,12	0,97	0,00	0,21

[a]*Insulated, plated and grown as described peripheral blood lymphocytes were stimulated with PHA (4 μg/ml), the tetanic toxoid (TT) and Tat (5 o 1 μg/ml) and tested as described in Table 4. Monkeys 1-3 were inoculated with 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of RIBI; 3 Monkeys 4-6 were inoculated or with 10 μg recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of Alum; two control monkeys were inoculated with RIBI (250 μl and 250 μl of autologous serum)(monkey 7) and with Alum (250 μl and 250 μl of autologous*

erum)(monkey 8).

Moreover, at week 15 five monkeys inoculated with the recombinant protein (monkeys 2-6) reacted to the skin test, further indicating the presence of a cell-mediated response enhanced by a strong retarded hypersensitivity reaction (Table 7 and figure 7).

TABLE 7.

Skin-test to Tat[a]

	Weeks from the beginning of immunisation	
Monkey N°	11	15
1	-	-
2	-	+
3	-	+
4	-	+
5	+	+
6	-	+
7	ND	ND
8	ND	ND

[a]*Tat (5, 1 and 0.2 μg) in 150 μl of PBS-0.1% BSA or its buffer were intradermally inoculated in a shaved area on the back of the vaccined animals, with the exclusion of the controls (ND, not determined) at week 11 and 15 after the first vaccine inoculation. The onset of an erythema after 24, 48 or 72 hours was considered a positive response.*

Such results show that the immunogen, and also the adjuvants RIBI and alum, were well tolerated by the animals and were non toxic, confirming the results referring to safety and tolerability of Tat vaccination obtained in the first pilot experiment. Moreover, such data confirm the finding reported in the first experiment that the Tat recombinant protein induces a strong specific anti-Tat umoral and cellular in vitro and in vivo.

Example 5. Co-stimulation with anti-CD3/28 beads of CD4+ lymphocytes purified from SIV+ infected monkeys induces the logarithmic expansion of the cells number in absence of significant virus replication and propagation.

The peripheral blood cells were depleted of the CD8 sub-population with anti-CD8 paramagnetic beads (Dynal, Oslo; Dynabeads M-450 CD8) and the purity of the obtained populations was evaluated through cytofluorimetric analysis and considered acceptable if higher than 95%. The depleted CD8 cells (called $CD8^-$PBMC) were cultivated and stimulated with PHA (2μg/ml) and IL-2 (40 U/ml) or with paramagnetic beads previously conjugated with two monoclonal antibodies specific for antigens CD3 (Clone FN18, Biosource) and CD28 (Clone 9.3, courtesy of Dr. Carl June) (anti-CD3/28 beads). To facilitate the bonding between anti-CD3/28 beads, the incubation occurred on a rotating device and the conjugated cells, selected with a magnet, were called CD8-CD3+CD28+ and cultivated. The cells concentration was brought back to the starting one three times per week and IL-2 was added where indicated; moreover, concerning the cells stimulated with anti-CD3/28 beads, preliminary experiments did show the convenience of utilising a continuous stimulation regimen, restoring the optimal beads/cells ratio at each counting. Our studies did show that in absence of exogenous IL-2 the CD8-CD3+CD28+ sub-population grows significantly better than the $CD8^-$PBMC cells, in response to the co-stimulation with anti-CD3/28 beads and that the addition of IL-2 (40 U/mL, 3 three times

per week) significantly enhances the proliferation kinetics referring both to duration and to obtained cells number (figure 8).

To evaluate the antiviral activity, CD8-CD3+CD28+ cells purified from 4 SIV$^-$ animals were infected at day 0 with 0.1 M.O.I. of SIV and maintained in continuous stimulation; as control CD8$^-$PBMC were used, stimulated with PHA and IL-2. The course of the infection was monitored by p27gag viral antigen determination in culture supernatants utilising a commercial ELISA kit (Coulter, Hialeah, FL). The p27 levels (ng/ml) measured at day 6 and 12 after infection and reported in Fig. 9 did show a sharp difference between the two stimulation condition in terms of infection productivity. In fact at day 6 the p27 percent reduction with reference to samples stimulated with PHA and IL-2 was comprised between 40% and 87% and at day 12 such reduction was enhanced in 2 out of 4 samples suggesting a lesser cells permissivity to the viral infection. Only in a case (mk 9401) we registered the infection propagation even with anti-CD3/28 beads stimulation. The described results show that *Macacus fascicularis* is a good model for the ex vivo expansion of lymphocitary sub-population in absence of significant viral replication through co-stimulation with anti-CD3/28 beads and is the basis for a therapeutic vaccine based on expansion and re-infusion, in HIV infected subjects, of autologous lymphocytes specific for the proposed viral antigens.

Prophetic example 6. Inoculation in *Maraca fascicularis* of a anti-Tat DNA vaccine.

The direct inoculation is proposed of pCV-Tat plasmide DNA, containing the cDNA of wild-type tat gene, and of pCV0 as control DNA. The plasmidic DNA to be administered to the animals are amplified in E.Coli bacteria (strain DH5) according to standard procedures [Molecular cloning - A Laboratory manual; Eds. Maniatis T., Fritsch E.F., Sambrook J., Cold Spring Harbor Laboratory, Cold Spring Harbor, New york (1992)] and according to protocols established by "European Agency for the evaluation of medicinal products; Human Medicine Evaluation Unit" (Technical Report Series No. 17 January 1997), purified by means of two CsCl gradients and dialysed for 48-72 hours against 100 volumes of sterile PBS (without Ca++ and Mg++). The DNAs are controlled through digestion with restriction enzymes, whose cut sites on plasmidic DNA are known, and their functionality is controlled through transfection of 5-10 μg of DNA with the calcium phosphate technique [Molecular cloning - A Laboratory Manual; Eds. Maniatis t., Fritsch E.F., Sambrook J., Cold Spring Harbor Laboratory, Cold Spring Harbor, New york (1992)] in H3T1 (1 x 10^6) cells, deriving from HeLa cells containing, integrated, a copy of the plasmide reporter HIV-1 LTR-CAT, and 48 hours after determination of the CAT enzyme [Gorman et al. Mol. Cell. Biol. 2:1044 (1982)].

The pCV-Tat or pCV0 DNAs (0.5-2 mg) resuspended in physiologic solution will be intramuscularly inoculated in two sites. 2-5 days before vaccination the animals will be inoculated with 1 ml of physiological solution containing bupivacaine 0.5% and metilparabene 0.1% in the two duly marked sited, in which later on the vaccination will be made, to increase the uptake and the DNA expression in the muscle [Danko et al., Vaccine 12:1499 (1994); Fine et al., Ann. Plast: Surg. 20:6 (1988)]. The monkeys will be inoculated at time 0 and after 5, 10, 15, 22 and 27 weeks. At week 32 a booster will be done with the recombinant Tat protein (10 μg) in presence of Iscom. The animals will be daily monitored as regards to the clinical parameters as described in example 4. Moreover, before the immunisation and in the same days of the inoculation, as described

in example 4, 10 ml blood samples will be taken. The protecting effect of the vaccination will be measured after the monkey challenge, which will be diminished at week 41 after beginning of the immunisation, intravenously inoculating 10 MID50 of SHIV. The post-challenge monitoring will be performed as described in example 4. Immunisation protocols will be also evaluated, in which there will be included combinations od DNAs expressing the other HIV-1 genes and/or cytokines, described in example 3.

The pCV0 and pCVTat plasmidic DNAs can also be inoculated in the animals utilising other delivery systems, which could make stronger the immunisation, such as the use of liposomes, nanoparticles or gene gun.

Prophetic example 7. Use of dendritic cells

The dendritic cells precursors will be isolated from peripheral blood of non-human primates of the Macaca *fascicularis* species through in vitro culture of adhering cells with GM-CSF and IL-4 for 7-14 days. The morphological analysis and the phenotypic characterisation (FACS analysis and immunoistochemistry after cytospin) will be made to confirm the identity of the cells obtanied in the culture. The functional analysis will be based on the capacity of the isolated cells to induce the proliferation of allogenic lymphocytes, peculiar characteristic of dendritic cells.

The dendritic cells resuspended at the $1x10^5$/100 µl concentration in RPMI 1640 containing 5% of autologous serum, 10 mM of Hepes buffer, 100 U/ml of penicilline-streptomicine, 0.5 mg/ml of amfotericine B, and glutamine at 0.03%, will be incubated for 2 hours at 37°C in presence of Tat or of its peptides or of the combinations Tat, Rev, Nef, Gag and/or cytokines and successively intravenously inoculated 2 or more times at intervals of 2-4 weeks. As an alternative, the dendritic cells will be transduced with vectors containing the tat gene alone or in combination with the other already mentioned vectors and subsequently intravenously inoculated.

Prophetic example 8. Therapeutic vaccination.

To define the efficiency of an anti-Tat therapeutic vaccination, both proteic and DNA, experiments will be made in monkeys already infected with SHIV in the asymptomatic and symptomatic phases. In particular, the onset of alteration in the inoculation site and of general symptoms in the asymptomatic monkeys, and of modification of the symptomatology in already symptomatic animals will be evaluated. In fact, an even remote probability exists that in infected monkeys the Tat inoculation will increase the viral replication, however such an effect should be a transitory one, considering both the low Tat inoculated dose and the short half-life of the transactivating protein (T1/2: 12 h). Should be the case, the Tat mutants will be used (in form of proteins peptides or DNAs), described in example 2. As an alternative, we will use in combination with the vaccine, inhibitors of the viral replication. To evaluate the vaccination effects on the illness progression, the monitoring of clinical, laboratory and viro-immunological parameters (above described) will be made at 0 time, at the moment of the next administration of the immunogen and 30 days after the same. After the last inoculation will take place monthly and each time a sudden change of the clinical situation will appear. PBMC, serum, plasma and urine samples will be frozen at each control for further evaluation as described before.

Prophetic example 9. Stimulation of the mucosal immunity

The described immunogens will be utilised to induce and/or enhance a specific immune response at mucose level. One of the routes will be used is based on the use of bacteria (Streptococcus gordonii, host in the oral cavity in humans) engineered to express the

above mentioned viral antigens. Such bacteria are able to colonise the oral and vaginal mucose in mice and to induce a specific, local and systemic, antibody response, with respect to eterologous antigens expressed on the recombinant bacteria surface [Oggioni et al., Vaccine 13: 775 (1995); Medaglini et al., Proc. Natl. Acad. Sci. USA 92: 6868 (1995); Medaglini et al., Vaccine 1997, in press]. The inventor believes that this method could be successfully utilised to induce also a specific vaginal immunisation in cynomolgus monkeys. As an alternative the mucosal immunity can be induced with the proteic immunogens above described, utilising other also bacterial delivery systems, such as cytofectines and liposomes, and those inoculation routes able to induce the better immune and protective response [Lewis et al., Vaccine Press, Ed. Robinson, Farrar, Wibling; Human Press, Totawa, New Jersey (1996); Lehnen et al., Vaccine Research 1:319 (1992); Honenbang et al., Infect. Immun. 62:15 (1994)].

Claims

1. Vaccine against AIDS tumours and syndromes recurring in HIV-1 infected subjects, comprising recombinant protein or peptides of the wild-type Tat and of the mutants thereof (cys22, lys41, RGDdelta and lys41-RGDdelta).
2. Vaccine according to claim 1, wherein the proteins or the peptides and mutants thereof are combined with Nef; Rev or Gag proteins or peptides.
3. Vaccine according to claim 1, wherein the proteins or the peptides and mutants thereof are combined with the IL-12 and IL-15 immunomodulating cytokines.
4. DNA vaccine against AIDS tumours and syndromes recurring in HIV-1 infected subjects, comprising DNA molecules or fragments codifying for wild- type Tat or for the mutants (cys22, lys41, RGDdelta and lys41-RGDdelta), said DAN molecules or fragments being inserted in the pCV0 plasmidic vector
5. Vaccine according to claim 4, wherein the DNA molecules or fragments are combined with DNA molecules or fragments of nef, rev and gag genes.
6. Vaccine according to claim 4, wherein the DNA molecules or fragments are combined with DNA molecules or fragments of IL -12 and IL -15 immunomodulating cytokines.
7. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or Nef, Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to immunise autologous dendritic cells induced to express the corresponding antigens.
8. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or Nef, Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to obtain drugs or vaccines for the immunisation of autologous dendritic cells transduced with eucariotic vectors containing said recombinant proteins or wild-type Tat and their mutants and combinations thereof.
9. Use according to claims 7 and 8 to produce drugs or vaccines for mucosal immunisation.
10. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or Nef, Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to obtain drugs or vaccines for the immunisation of peripheral blood cells through co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies.

Rome, 1 Dec. 1997

f. Barbara Ensoli

The Representative

(signature)

Dr. Maria Vittoria Primiceri

of NOTARBARTOLO & GERVASI S.p.A.

FORM A

DUTY STAMP

TO THE MINISTRY OF INDUSTRY COMMERCE AND HANDICRAFT
Main Patent Office - ROME
Patent Application for Industrial Invention, filing of reserves,
advanced opening to public inspection

A. Applicant (1) G.S. Physical person
1) Name Ensoli Barbara
Residence Via Festo Avieno 176 – 00136 ROMA code NSLBBR60A63E472K
2) Name G.S.
Residence code

B. APPLICANT'S REPRESENTATIVE BEFORE M.P.O.
Surname, name Maria Vittoria PRIMICERI et al Fiscal code
Name of the corresponding office NOTARBARTOLO & GERVASI S.p.A.
Address Via Savoia 82 city ROME zip code 00198 province RM

C. ELECTED DOMICILE OF THE ADDRESSEE As above
Address city code prov

D. TITLE proposed class, (sec./cl./ucl.) group/subgroup

HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes

ADVANCED OPENING TO PUBLIC INSPECTION yes___ no____
in presence of amendment request: date no. of ref.:

E. NAMED INVENTORS

surname, name	surname, name
1) Barbara Ensoli	3)
2)	4)

F. PRIORITY
Country or Exhibition Type of Priority Appln. No. Appln. date Encl(yes/res)

1) NONE

2)

G. CENTRE FOR COLLECTING MICROORGANISMS'CULTURES, denomination

None

H. SPECIAL NOTES
None

ENCLOSED DOCUMENTS

Specimen No.				RESERVES DISSOLUTION
				date No. of ref.
Doc. 1) 1 prov.	no. sheets 24	abstract with main drawing, spec. and claims (compulsory 1 copy)		30 JAN 1998 RMR0091
Doc. 2) 1 prov.	no. sheets 09	(compulsory if cited in description., 1 copy)		30 JAN 1998 RMR0091
Doc. 3) 1 res.		power of attorney or reference attorney		
Doc. 4) 0 res.		designation of inventor		
Doc. 5) 0 res.		priority document with Italian translation		comparison single priority
Doc. 6) 0 res.		authorisation or assignment deed		
Doc. 7) 0 res.		complete name of the applicant		

8) PAYMENT RECEIPT OF LIT. 565.000.= compulsory
filled in on 01.12.1997

The applicant's signature Dr. Maria Vittoria Primiceri
(signature)

follows yes/no no
We required certified copy of the present deed yes/no yes

PROVINCIAL OFFICE OF INDUSTRY COMMERCE HANDICRAFT OF ROME code 58
FILING CERTIFICATE Application no. RM97A000743 Reg. A
The year 1997 the 1st day of the month of December

The above mentioned applicant(s) has(have) presented to me undersigned the present application consisting of no. 00 additional sheets for the grant of the above patent.

I. DIFFERENT NOTES OF THE RECORDING OFFICER
none

THE DEPOSITER
(signature)

THE RECORDING OFFICER
(signature)
SEAL

Description of the patent application for industrial invention having for title:

"HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes"

in the name of Barbara Ensoli

residing in Rome

named inventor: Barbara Ensoli

RMR0091

*****

The present invention refers to a vaccine, prophylactic or therapeutic, anti-HIV, anti AIDS and against tumours and syndromes associated to HIV infections utilising proteins, peptides and DNA (wild-type or mutant) of HIV Tat, alone or associated to proteins, peptides and DNA of other viral products (Nef, Rev, Gag) or of cytochines having an enhancing action on antiviral immune response.

The invention refers also to the immuniization by means of otology, dendritic cells, mucosal immunization or ex-vivo immunisation of peripheral blood cells expanded through co-stimulation with monoclonal antibodies anti-CD3 and anti-CD28.

Background of invention

AIDS (acquired immunodeficiency syndrome) is caused by HIV virus and characterised by immunodeficiency, tumours, mainly Kaposi sarcoma (KS) and B-cells lymphomas, opportunistic infections and central nervous system alterations. Since AIDS is world-wide spread and has a high mortality, one of the most important public health goals is a prophylactic and/or therapeutical anti-HIV vaccine. Therefore it was mainly utilised as immunogen the viral envelope or sub-units thereof, but with unsatisfactory result due to the extreme variability of the viral envelope. [Wain-Hobson, Curr. Opin. Genet. Dev. 3:878 (1993); Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics, Los Alamos, NH, 1995)]. Therefore it is common opinion that, as an alternative to sterilising immunity, it could be sufficient to have the infection progression stop (therapeutical vaccine). Moreover, immunoprotective responses can be obtained utilising as immunogens

giones of pathogenic agent [Lu et al., J. Virol. 70:3978 (1996); Boyer et al., Nature Med. 3:526 (1997)]. Owing to the published experimental data, the inventor thinks it necessary to utilise a vaccine produced with the most stable viral products other than env, between those isolated from HIV, inducing an effective both umoral and cellular response, and having a vital function for the virus.

Such products must be experimented in the model of non human primates in that their immune system is more similar to the human one than that of philogenetically more distant animals and in which IDS develops after infection. HIV-1 Tat regulating protein has all such characteristics: it is stable, immunogenic and essential for the early phases of the viral infection. Moreover, Tat has the fundamental role not only in the viral replication and in infection transmission and progression, but also has a starting and progression factor of AIDS associated tumours, for instance KS, which is the most frequently AIDS associated tumour and of other syndromes and syntoms developing after HIV infection.

Tat is a protein of 86-102 amino acids, depending on the viral strain, codified by two exons. Tat is produced soon after the infection, localises itself in the nucleus and transactivates the expression of the viral genes interacting with the target sequence "Tat-responsive element" (TAR) of LTR [Chang et al., J. Biomed. Sci. 2:189 (1995)]. Tat could also have a role in the HIV virulence through other action mechanisms in the viral cycle [Huang et al., EMBO J. 13:2886 (1994); Neuvet et al., J. Virol. 70:5572 (1996); Harrich et al., EMBO J. 16:6 (1997); Li et al., Proc. Natl. Acad. Sci. USA 94:8116 (1997)]. The product of the first exon (amino acids 1-72) is preserved in different viral extracts [Myers et al., Human retroviruses and AIDS. Theoretical Biology and Biophysics. Los Alamos. (1995)] and is sufficient for the transactivation

of HIV-1 [Chang et al., J. Biomed. Sci. 2:189 (1995)]. It contains 4 domains. The acidic domain (amino acids 1-21) is important for the Tat interaction with cellular proteins; the cysteine reach region (amino acids 22-37) represents the transactivation domain. This region is the more preserved one in the primary extracts [Meyerhans et al., Cell 58:901 (1989)]. The substitution of cysteine 22 with a glycine cancels the Tat capacity of transactivating the HIV-LTR [Yang et al., J. Virol. 70:4576 (1996)]; the core region (amino acids 38-48) is also preserved and is important for the activation and substitution of lysine 41 with a threonine, inactivates the transactivating activity of Tat on HIV LTR [Kashanchi et al., J. Virol. 70:5503 (1996)]; the basic domain (amino acids 49-57), reach in arginine and lysine, is necessary for the nuclear localisation and specifically bonds its target RNA (TAR) [Chang et al., J. Biomed. Sci. 2:189 (1995)]. Moreover, the basic region is responsible for the bonding of extracellular Tat to the eparine and to the proteoglycan-eparansulphates (HSPG) [Chang et al., AIDS 11:1421 (1997)]. Mutation in the basic region cancels such interactions. The carboxyl terminal portion of Tat is not necessary for the LTR transactivation, but contains a argynine-glycine-aspartic acid sequence (RGD), usually present in the extracellular matrix proteins (ECM), responsible for the Tat bonding to integrinic receptors $\alpha_5\beta_1$ e $\alpha_v\beta_3$ and for the Tat effects on AIDS associated tumours and on immune, vascular and nervous system [Barillari et al., Proc. Natl. Acad. Sci. USA 149:3727 (1993); Ensoli et al., Nature 371:674 (1994); Zauli et al., Blood 80:3036 (1996); Chang et. al., J. Biomed. Sci. 2: 189 (1995)]. During the acute infection of T-cells with HIV-1 or after the transfection of tat gene in COS-1 cells, the Tat protein is released in absence of cellular death in the extracellular environment [Ensoli et al., Nature 345:84 (1990)].

Ensoli et al., J. Virol. 67:277 (1993); Chang et al., J. Biomed. Sci. 2:189 (1995)]. The Tat release from infected cells occurs also in vivo since Tat is present in infected subjects serum [Westendrop et al., Nature 375:497 (1995)] and in the AIDS-KS lesions [Ensoli et al., Nature 371:674 (1994)]. After the release, part of the protein remains in a soluble form, while the other one links itself to the HSPG of ECM. Tat linked to the HSPG can be recovered in an eparine soluble form. The bond with eparine is due to the Tat basic region, prevents the effects of the extracellular Tat and protects the protein fro the oxidation, to the point that it permitted us the purification of high biological activity Tat [Chang et al., AIDS 11:1421 (1997)]. Extracellular Tat can be absorbed by the cells, can migrate in the nucleus and can transactivate the viral genome expression [Frankel et al., Cell 55:1189 (1988); Mann et al., EMBO J. 10:1733 (1991); Marcuzzi et al., J. Virol. 66:4228 (1992); Ensoli et al., J. Virol. 67:277 (1993)]. The absorption of That occurs by endocytosys mediated by the RGD bond to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [Barillari et al., Proc. Natl. Acad. Sci. USA 90:7941 (1993) Ensoli et al., Nature 371:674 (1994)] and/or through endocytosys mediated by the basic region bonding to HSPG.

Tat can activate the viral replication and the infection diffusion also through indirect mechanisms involving the modulation of the expression of cellular genes which play a key role in the cells survival control, and of inflammatory cytochines action on viral replication [Chang et al., J. Biomed. Sci. 2:189 (1995)].

Beyond its importance in the viral replication, Tat plays a very important role in the AIDS pathogenesis. That is able to modulate the survival and proliferation of infected and non-infected cells by causing activation or repression of cytochines, such as IL-2 [Puri et al., AIDS Res. 11:31 (1995); Westendorp et al., J. Virol.

68:4177 (1994); Chirmule et al., J. Virol. 69:492 (1995)], or of genes having a key role in the cellular cycle [Sharma et al., Biochem. Biophys. Res. Co. 208:704 (1995); Zauli et al., Blood 86:3823 (1995); Li et al., Science 268:229 (1995); Westendorp et al., Nature 375:497 (1995); Gibellini et al., Blood 89:1654 (1997)]. The anti- or pro-apoptotic effects of Tat depend on a number of factors such as cellular type, the fact that Tat is expressed from the cell or added to the cell and from its concentration [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Zauli et al., J.Immunol. 157:2216 (1996)].

Tat is the factor responsible for the enhanced frequency and aggressiveness of KS in HIV-1 infected subjects [Ensoli et al., AIDS Updates, Eds. V. De Vita, Jr., Hellman S., Rosenberg S.A., Lippincott J.B., Philadelphia; 7: 1 (1994); Corallini et al., Cancer Res. 53: 1 (1993)]. KS is a vascular originating tumour and the most frequent neoplasia in virus infected subjects. Tat induces the KS cells and the endothelial cells activated by inflammatory cytochines (IC) to migrate, to express collagenasys IV, to invade ECM and to proliferate, such processes being necessary for the angiogenesys and the tumoral invasion. [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994); Albini et al., Proc. Natl. Acad. Sci. USA 92:4838 (1995); Fiorelli et al., J. Clin. Invest. 95:1723 (1995)]. Such Tat effects are induced by IC, in that they stimulate the Tat receptors expression, l'$\alpha_5\beta_1$ and $\alpha_v\beta_3$ [Barillari et al., Proc. Natl. Acad. Sci. 90: 7941 (1993)]. Tat mimes the ECM proteins effect, such as fibronectine and vitronectine and the region containing RGD and the basic region are necessary for the effects of the extracellular Tat on KS cells, on angiogenesys and on progression of KS. The evidence of extracellular Tat presence and of its in vivo bond to the Tat receptors in

the AIDS-KS lesions [Ensoli et al., Nature 345: 84, 1994] supports the idea that Tat is involved in the onset and the manteinment of the KA associated to AIDS. Moreover gene tat for transgenic rats develop KS or other phenotype tumours depending on the expression level of the transgene [Vogel et al., Nature 335: 601 (1988); Corallini et al., Cancer Res, 53: 5569 (1993)].

It was suggested that Tat plays o role in the iperproliferative phenomena and in the pathogenesis of the B lymphomas, frequently observed in sieropositive subjects and in tat transgenic rats [Vellutini et al., AIDS Res. Hum. Retrov. 11:21 (1995)], through mechanisms involving the enhancement of bcl-2 and cytochines expression [Puri et al., Cancer Res., 52:3787 (1992)]. Other evidences confirm a probable role of Tat in oncogenesis [Kim et al., Oncogene 7: 1525 (1992)].

Tat can also activate the expression of viral promoters, such as those of the herpesviruses and of other viruses which reactivate themselves in AIDS subjects, promoting the onset and progression of opportunistic infections [Chang et al., J. Biomed. Sci. 2:189 (1995)].

Tat seams also able to exert neurotoxic effects both direct (through the basic region and the RGD, and indirect through induction of inflammatory cytochines having a toxic effect on the neurones of the central nervous system or on the ematoenchephalic barrier [(Chang et al., J. Biomed. Sci. 2:189 (1995)]. In adult and paediatric subjects the central nervous system alterations can lead to dementia and to learning and behavioural alterations.

As far as the Tat immune response is concerned a number a studies suggest that anti-Tat antibodies play a protective role and can control the illness evolution in vivo [Reiss et al., J. Med Virol. 30:163 (1990); Rodman et al., Proc. Natl. Acad. Sci. USA

90:7719 (1993); Rodman et al., J. Exp. Med. 175:1247 (1992); Re et al., J. Acquir. Immun. Defic. Syndr. 10:408 (1996)]. Moreover, in vitro, anti-Tat antibodies not only suppress the absorption, the transcellular activation of Tat and the infection [Ensoli et al., J. Virol. 67:277 (1993); Re et al., J. Acq. Immun. Def. Synd. 10:408 (1995)], but also in inhibit the AIDS-KS cells proliferation and Tat induced migration and the formation of KS-like lesions in rats [Ensoli et al., Nature 345:84 (1990); Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994)]. Moreover, our preliminary results show that anti-Tat antibodies are absent in AIDS-KS subjects, suggesting that such subjects do not have antibody activity blocking the extracellular Tat and, hence, that this protein can act undisturbed.

The development of an anti-Tat cell-mediated response in the initial phase of the infection is important for the control of the infection itself [Voss et al., Virology 208:770 (1995); Rinaldo et al., AIDS Res. Hum. Retrov. 11:481 (1995); Harrer et al., AIDS Res. Hum. Retrov 12:585 (1996)] and there exists an inverse correlation between the specific anti-Tat CTL presence and illness progression [van Baal et., J. Gen. Virol. 78:1913 (1997)]. Such results were obtained in studies on macaques inoculated with SIVmac [Lu et al., J. Virol. 70:3978 (1996); Venet et al., J. Immunol. 148:2899 (1992)]. Moreover, recent works on rats of different species in which Tat was inoculated both as plasmide and as protein did show that it is possible to induce both a umoral and cellular response towards the protein [Hinkula et al., J. Virol. 71:5528 (1997)] though it was observed a variability between rat species and that such results were not been reproduced with the same immunogens in a work on non-human primates [Quesada-Rolander et al., ABS 6-S1, 2nd European Conference on Experimental AIDS Research, Stockholm, Sweden, May 31-June 3

(1997)]. The lack of reproducibility in the vaccine experimentation in rats with respect to the one on non-human primates is frequent and possibly due to the different immune system in the two animal species which can bring to different immune responses with the same immunogen, as demonstrated for HIV with the virus Env protein. All that requires that the immunogens proposed for vaccine experimentation for men must tested on non-human primates and not only in inferior species. The inventor believes that other viral proteins, or parts thereof, could be associated with Tat and could enhance an immune response specific to HIV and could be of benefit also in the vaccination against onset of tumours and of other pathologies and symptoms associated to HIV infection. Such products are the HIV Nef, Rev and Gag proteins.

Nef is another viral regulating protein important for the development of the illness [(Allan et al., Science 230:813 (1985); Franchini et al., Virology 155:593 (1986); Guy et al., Nature 330:266 (1987)] early produced after the infection and released in the extracellular environment. In the system SIVmac/macaque the presence of Nef is correlated with high doses of viral amount and with progression towards AIDS [(Kestler et al., Science 248:1109 (1991)]. Nef is more variable than Tat [(Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics. Los Alamos, NH (1995)]. Nef is an immunogenic protein [(Gobert et al., Virology 176:458 (1990); Choppin et al., J. Immunol. 147:569 (1991); Culman et al., J. Immunol. 146:1560 (1991); Tahtinen et al., Virology 187:156 (1992)], and it is able to induce CTL [(Bourgault et al., J. Virol. 66:75 (1992); Couillin et al., J. Exp. Med. 180:1129 (1994)]. In particular, it was pointed out an immunodominant region in the Nef central region (region 73-144) which is recognised in the largest part of patients with anti-

Nef CTL.

Rev is a viral regulating protein early produced during infection [Gait et al., Trends Biochem. Sci. 18:255 (1993); Parslow, Human Retroviruses, Ed. B.R. Cullen, IRL press, Oxford, England, p. 101 (1993)] and released in the extracellular environment. Rev is essential for the HIV replication and for the illness progression, and is codified by two exons, partially superimposed to the ones codifying for That. Rev is a nuclear protein [Felber et al., Proc. Natl. Acad. Sci. 86:1495 (1989)] necessary for the expression of the RNAs viral messengers for the tardy proteins [Malim et al., Nature 338:254 (1989)]. Rev is a highly preserved protein in the various viral extracts of HIV-1 [Meyers et al., Human Retroviruses and AIDS: A compilation and analysis of nucleic acid and amino acid sequences, Los Alamos Laboratory, Los Alamos, NM p.1 (1993)] and it is immunogenic. In fact it induces the production of specific antibodies directed against the two functional domains of the protein [Pilkington et al., Mol. Immunol. 33:439 (1996)] during the natural infection in man [Reiss et al., AIDS Res. Hum. Retrov. 5:621 (1989)] and after inoculation in rats [Hinkula et al., J. Virol. 71:5528 (1997)]. The lowering of anti-Rev antibodies seral levels seems to be correlated with the progression towards AIDS [Reiss et al., AIDS Res. Hum. Retrov. 5:621 (1989)]. Rev can induce CTL both in men and in monkeys [van Baalen et al., J. Gen. Virol. 78:1913 (1997); Venet et al., J. Immunol. 148:2899 (1992)] and it was reported that a specific anti-Rev CTL response, early during the infection is inversely correlated with the illness progression [van Baalen et al., J. Gen. Virol. 78:1913 (1997); Venet et al., J. Immunol. 148:2899 (1992)].

Another viral target is the gag gene, which is tardily expressed during infection and codifies for a group of highly immunogenic structural capsidic proteins [Bruisten et

al., J. Infect. Dis. 166:620 (1992); Sipsas et al., J. Clin. Invest. 99:752 (1997)]. The anti-Gag antibody titres maintain themselves high and stable during the asymptomatic phase of the infection, and reach very low levels when the infection goes on to clear AIDS, in combination with the CD4+ lymphocites drop and the finding of the virus in the blood [Baur et al., J. Infect. Dis. 165:419 (1992); Koup et al., J. Virol. 68:4650 (1994)]. Gag protein induce CTL activity early during infection, both in men and in primates [Mcfarland et al., J. Inf. Dis. 170:766 (1994); Yasutomi et al., J. Virol. 70:678 (1996)], and their presence is significantly related with the control of initial viremia and with the illness progress [Klein et al., J. Exp. Med. 181:1365 (1995); Aryoshi et al., AIDS 9:555 (1995); Rinaldo et al., J. Virol., 69:5838 (1995); Yang et al., J. Virol. 70:5799 (1996); Lubaki et al., J. Infect. Dis. 175:1360 (1997)]. Moreover, proteins p17 and p24 contain immunodominant epitopes which are maintained in different extracts of HIV-1 and HIV-2 and recognised by CTL [Littaua et al., J. Virol. 65:40 (1991); Buseyne et al., J. Virol. 67:694 (1993); Nietfield et al., J. Immunol. 154:2189 (1995); van Baalen et al., J. Gen. Virol., 77:1659 (1996); Nixon et al., Nature 336:484 (1988)].

The inventor believes that cytochines or parts thereof, such asIL-12 and IL-15, or other immunomodulant cytochines or in any case enhancing the immunogenic effect, can be utilised as adjuvant in the anti-Tat vaccination. IL-12 is a strong immunoregulatory cytochine produced by cells having the antigen such as B and dendritic cells [Per review see Trinchieri, Curr. Opin. Hematol. 4:59 (1997)]. IL-12 is early produced after HIV infection and has a pro-inflammatory action inducing NK cells and T lymphocytes to produce IFNγ which makes the phagocytes activated and promotes the Th1 lymphocytes induction. IL-12 plays a fundamental role in rising the

resistance to a number of infections caused by bacteria, fungi, viruses and shows high antitumoral activity. It is believed that the immunodepression inducing viruses such as HIV and measles virus, act also through IL-12 production depressing mechanisms. [Grosjean et al, J. Exp. Med. 186: 801 (1997); Fugier-Vivier et al, J. Exp. Med. 186: 813 (1997) Schnorr et al, Proc. Natl. Acad. Sci. USA. 94: 5326 (1997)].

IL-15 is a pleiotropic cytochine expressed by non-lymphoid tissues, by activated monocytes/macrophages and by dendritic cells[Quinn et al., Biochem. Biophys. Res. Commun. 239:6 (1997); Jonuleit et al., J. Immunol. 158:2610 (1997)]. IL-15 plays an important role in regulating the NK activity, in the lymphocytes T proliferation and in the CTL activity [Jullien et al., J. Immunol. 158:800 (1997); Carson et al., J. Clin. Invest. 99:937 (1997)]. IL-15 induces the expression of CTL against HIV antigens, and in IL-2 absence and T CD4+ functional lymphocytes [Kanai et al., J. Immunol. 157:3681 (1996); Agostini et al., Blood 90:1115 (1997)]. Moreover, IL-15 as IL-2, induces the onset of lymphocytes having cytotoxic activity ("lymphokine-activated killer", LAK) and stimulates the IFNγ production in PBMCs of seropositive patients [Lucey et al., Clin. Diagn. Lab. Immunol. 4:43 (1997)]. IL-15 activated the monocytes to produce chemokines, playing a controlling role in the onset of inflammatory processes [Badolato et al., Blood 90:2804 (1997)].

Within the different systems aimed at the generation of effective antiviral and antitumoral vaccines the inventor thinks that utilisation of dendritic cells could be a key element in the induction of the Tat immune response. This is due to the fact that those are the most efficient cells in presenting the antigen and the sole able to stimulate intact lymphocytes, adjuvants being absent [Steinman R.M., Exp. He[illegible]

24: 859 (1996)]. The use of dendritic cells replaces the function of a number of adjuvants consisting in the induction of a non specific immunitary response(natural immunity) which in turns generates a potent primary specific response in presence of the antigen.

Since the transmission of HIV infection primarily occurs at mucosal level (genital and rettal in the adult, oral in the new-born), the inventor thinks that the induction of protective immunity at mucosal level is a primary important goal, many works did recently show the possibility to induce local and systemic immunisation. Particularly the nasal and oral route did show to be the most efficient in inducing an effective mucosal immunitary response, even in distant sites, such as genital mucosas [Rosenthal et al., Seminars in Immunology 9:303 (1997); O'Hagan et al., Novel Delivery Systems for Oral Vaccines, Eds. O'Hagan, D.T. CRC Press Boca Raton, FL, p. 176 (1994)].

Recent woks did show that the co-stimulation of CD4+ lymphocytes with paramagnetic beads coated with anti-CD3 e anti-CD28 monoclonal antibodies determines the logarithmic and polyclonal expansion of the lymphocytes coming from HIV-infected subjects [Levine et al., Science. 272: 1939-1943 (1996)] without activating the replication and propagation of the virus. Such antiviral activity is a consequence of the negative modulation of the expression of CCR5 , co-receptor of HIV-1 monocytotropic strains [Carrol et al, Science. 276: 273-276, (1997)] and, in a lesser extent, to the high levels of chemokines induced by co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies [Riley et al, J.Immunol. 158: 5545-5553, (1997)]. The inventor thinks that the possibility to expand autologous lymphocytes from HIV infected subjects in absence of viral replication/propagation, permits to

obtain an effective ex vivo immunisation, described in the examples, which can be highly helpful in producing an anti-Tat vaccine.

The inventor thinks that such observations suggest that the immunisation with Tat, alone or in combination with other viral products or immunomodulant cytokines, or parts thereof, could stop the viral replication in subjects exposed after vaccination and in already infected subjects, maintaining the infection in an abortive phase, which therefore could be more easier controlled by the immune system. Therefore the inventor thinks that a Tat based vaccine should be able to induce an immunitary response, both umoral and cellular, sufficient to stop or reduce the replication or the transmission of the virus and therefore to protect from the infection, from the illness and from the onset of the tumours and of the other syndromes and symptoms associated with AIDS. It is therefore possible to use the anti-Tat vaccine both as prevention as therapy. In fact a umoral response against Tat could neutralise the effects of extracellular Tat reducing and limiting the infection, while the cell-induced response against Tat and other viral proteins, enclosed in the vaccine formulation, should destroy the virus replicant cells and therefore to control the infection and to permit to the immune system of the host to develop a complete immune response towards the viral components in absence irreversible damages due to the viral replication.

Claims

The present invention refers to:

1. a prophylactic and therapeutic proteic or peptidic vaccine to be used in men, to be used against AIDS, tumours and syndromes and symptoms associated to HIV infection, comprising recombinant proteins of wild-type Tat and mutants thereof

(Seq. 1-5), expressed and purified as described, or of its wild-type or mutant peptides (Pep. 1-7), to be administrated alone or combined with the universal T-helper epitope of tetanic toxoid or other T-helper peptides;

2. a vaccine as described in the above, combined with recombinant proteins or with Nef, Rev or Gag peptides of HIV, or administrated in form of Tat/Nef, Tat/Rev, Tat/Gag fusion proteins or part thereof;

3. a vaccine as described in the above, in combination with recombinant proteins of immunomodulating cytokines such as IL-12, IL-15 or other molecules or parts thereof, enhancing the antiviral immune response, or administrated as Tat/IL12, Tat/IL-15 or Tat/other molecules fusion proteins, or parts thereof, enhancing the antiviral immune response;

4. a prophylactic and therapeutic vaccine to be used in men, against AIDS, tumours and syndromes or symptoms associated with HIV infection, comprising vectors which codify Tat wild- type or relevant mutants (Seq. 1-5), or parts thereof, inserted in the PCVO plasmidic vector other vectors;

5. a DNA vaccine as described at point 4, in combination with DNA molecules inserted in the pCV0 plasmidic vector or other vectors, of HIV rev, nef e gag genes or parts thereof, or administrated as plasmids co-expressing tat/rev, tat/nef, tat/gag or parts thereof;

6. a DNA vaccine as described in the above, in combination DNA molecules based on pCV0 plasmid or other vectors, of the genes for the immunomodulating cytokines IL-12, IL-15 or other immunomodulating genes, or parts thereof, enhancing the antiviral immune response, or administrated as plasmid co-expressing tat/IL-12, tat/IL-15 or tat/other molecules, or parts thereof, able to

enhance the antiviral immune response;

7. a proteic, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the immunisation with autologue dendritic cells by means of ex vivo treatment;
8. a proteic, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the mucosal immunisation (nasal, oral, vaginal or rettal);
9. a protein, peptidic and/or DNA anti-Tat vaccine alone or combined as above, described for the immunisation ex vivo of infected subjects peripheral blood cells expanded though co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies and re-infused in the host;
10. a proteic, peptidic and/or DNA anti-Tat vaccine as described, combined with viral replication inhibitors.

Detailed description of the invention

The present invention relates to a prophylactic and therapeutic vaccine against the HIV infection, the progression towards AIDS, the developments of tumours and of other syndromes and symptoms in subjects infected by the virus, utilising recombinant, proteic, peptidic and DNA- molecules of HIV-Tat. More particularly the invention refers to a vaccine based on HIV-1 Tat as immunogen, inoculated as DNA and/or recombinant protein or of peptides, alone or in combination with other genes or viral genetic products (Nef, Rev, Gag) or parts thereof, or in combination with various immunomodulating cytokines (IL-12, IL-15) or with codifying gene for an immunomodulating cytokine or part thereof. Tat, Nef, Rev, Gag and the immunomodulating cytokines are administrated both as a mixture of recombinant proteins, of peptides or of fusion proteins (Tat/Nef, Tat/Rev, Tat/Gag, Tat/IL-12,

Tat/IL-15) and as plasmidic DNA. It is also described an immunisation method utilising autologous dendritic cells treated ex vivo with recombinant Tat protein, or peptides thereof, alone or with a mixture of recombinant proteins or peptides (Tat, Nef, Rev, Gag) or with the Tat protein and one or more immunomodulating cytokines, or parts thereof, or transduced with eucariotic vectors containing Tat alone or with codifying viral genes for Nef, Gag or Rev, or Tat and the codifying gene for an immunomodulating cytokine or part thereof.

It is also described the induction use of an immune response at the mucosal level. Tat or its peptides, alone or in combination with viral proteins and/or citokines is inoculated also at mucosal level to enhance and induce the local immunitary response. The HIV- Tat protein or sub-units thereof will also be utilised for the *ex vivo* immunisation of CD4+ and CD8+ lymphocytes insulated from the peripheral blood of infected subjects. Later the Tat antigen specific cells will be expanded in vitro through co-stimulation with monoclonal antibodies directed against CD3 and CD28 and re-infused In is also described the use of Tat mutants, identified in the examples, to be utilised as immunogens, as an alternative to Tat wild type. The Tat mutants are i) in the cysteine region (cys22) and ii) in the core region (lys41), iii) the deleted mutant in the RGD sequence; iv) the double mutant in lysine 41 and in RGD. Alternatively to the use of Tat mutants or Tat peptides (wild type or mutated as the protein) in case of therapeutic vaccination, along with the immunogen, inhibitors of the viral replication will be utilised.

The present invention will be now described by means of its illustrative and not limitative specific examples, in which reference will be made to the enclosed figures.

Brief description of the figures

FIG. 1. CAT test to determine the capability of purified Tat-cys22 (Tat22) protein to compete with the transactivating activity of Tat wild type protein. H3T1 cells, containing the HIV-1 LTR-CAT "reporter" vector integrated in its genome [Sodroski et al., Science 227:171, (1985)], in which the gene for the chloramphenicol acetyl-transferase enzyme (CAT) is placed under transcriptional control of the LTR promoter of HIV-1, have been incubated in presence of Tat wild type protein (100 ng) alone or in combination with a molar excess of Tat-cys22 protein (1 μg). The transactivating activity of Tat on the HIV-1 LTR and the capability of the Tat-cys22 protein to compete with Tat wild type have been determined after 48 h, by subjecting amounts of the cytoplasmic extracts (corresponding to 200 μg of protein) to the assay for determination of the activity of the cAT enzyme, as described [Ensoli et al., J. Virol. 67:277 (1993)]. The percentages (%) in acetylation of ^{14}C-chloramphenicol are indicated.

FIG. 2 . Umoral response of anti-Tat specific IgG type in monkeys vaccinated with the Tat protein, determined by immuno-enzymatic assays (ELISA). (A) shows the results obtained on two monkeys inoculated with 10 or 100 μg of Tat recombinant protein re-suspended in 250 μl autologous serum and 250 μl RIBI, subcutaneously in a site; (B) shows the results for the non-inoculated control monkey. Monkeys were inoculated at 0 time and after 2, 5, 10, 15, 22 and 27 weeks. The presence of the anti-Tat antibody response in the vaccinated animals plasma was evaluated by ELISA assays prepared and characterised by us. The Tat protein was adsorbed at 96 well plates in PVC (100 ng/well in 200 μl carbonate buffer 0.05 M pH 9.6) for 12 h at 4°C. After 3 washings with PBS-A 1x containing Tween 20 (0.05%), sera

added (in duplicate) diluted 1:50 in 200 µl carbonate buffer, it was incubated at 37°C for 90', the wells were washed with PBS-A 1x/Tween 0.05% and the presence of the immunocomplexes was highlighted by adding 100 µl of secondary antibody (diluted 1:1000 in PBS-A 1x/Tween 0.1%/BSA 1%) conjugated with horseradish peroxidase, for 90' at room temperature. After 5 washings of the wells, 100 µl of substrate of the peroxidase (ABTS 1 mM, Amersham) were added for 30-45' at room temperature and then a spectrophotometric reading was carried out at 405 nm. Each ELISA essay included a polyclonal serum included anti-Tat rabbit polyclonal serum (positive control) diluted 1:200 to 1:6400, and the monkeys sera taken at 0 time (negative control) diluted 1:50. The cut off value was considered as (± S.D.) mean of the readings of the sera of all monkeys, taken at 0 time, obtained in all the experiments. The results shown by the histogram for each sample correspond to the mean optical densities at 405 nm of the readings of the two wells, subtracted of the cut off value ± S.D (ΔOD405). >2,7: The read value were out of scale.

FIG. 3. Titration of the plasma of monkeys inoculated with 100 and 10 µg recombinant Tat protein, described in fig. 2. The results in ordinate are represented as the inverse of the highest dilution of the serum which at 405 nm had a reading higher than the cut off value.

FIG. 4. Analysis of the specific anti-Tat umoral response of IgM type in monkeys inoculated with Tat and determined by ELISA. essay. Three monkeys (M1-3) subcutaneously inoculated with 10 µg recombinant Tat protein re-suspended in 250 µl autologous serum and 250 µl RIBI and 3 monkeys (M4-6) subcutaneously inoculated with 10 µg recombinant Tat re-suspended 250 µl autologous serum and

250 µl Alum; 2 control monkeys subcutaneously inoculated with RIBI (250 µl and 250 µl autologous serum) (M7) and with Alum (250 µl and 250 µl autologous serum) (M8). The monkeys were inoculated at 0 time and after 2, 6, 11 and 15 weeks. The ELISA essays were performed and the cut off values were determined as described in fig. 2. In this case the sera of the animals were tested (in duplicate) at dilution 1:100 and monkey anti-IgM goat serum conjugated with horseradish peroxidase was used as secondary antibody, diluted at 1:1000.

FIG. 5. Analysis of the umoral response of anti-Tat specific IgG type in monkeys inoculated with Tat tested by ELISA. Three monkeys (M1-3) inoculated with 10 µg of recombinant Tat re-suspended in 250 µl autologous serum and 250 µl RIBI and 3 monkeys (M4-6) inoculated with 10 µg recombinant Tat resuspended in 250 µl autologous serum and 250 µl Alum; two control monkeys inoculated with RIBI (250 µl and 250 µl autologous serum) (M7) or with Alum (250 µl and 250 µl autologous serum) (M8). The monkeys were inoculated at 0 time and after 2, 6, 11 and 15 weeks. The ELISA essays were carried out and the cut off values were determined as described in fig. 2. >2,7: the value was out of scale.

FIG. 6. Titration of the sera of the monkeys inoculated with recombinant Tat (10 µg) in presence of RIBI adjuvant (M1-3) or Alum (M4-6). The results are shown for each serum as the inverse of the highest serum dilution which at 405 nm gave a reading higher the cut off value.

FIG. 7. Analysis of the response of delayed ipersensitivity to Tat by performing skin test. Tat protein (5, 1 and 0.2 µg), re-suspended in 150 µl PBS containing 0.1% BSA and the buffer in which Tat is resuspended were intradermally inoculated in

shaved area on the animal back. The area was photographed at 0 time and after 24, 48 and 72 hours. The control monkeys were inoculated only with buffer. In the figure it is shown an example corresponding to the 15th week for the M2 monkey at 48 h after the inoculation of the immunogens. It is evident a strong positive reaction to Tat.

FIG. 8. Kinetic of the PBMC proliferative response of *Macaca fascicularis* to the co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies on paramagnetic beads (anti-CD3/28 beads). The PBMC insulated from peripheral blood were depleted of the CD8 positive sub-population by immunomagnetic methods. Afterwards, part of anti CD8-depleted lymphocytes was stimulated with PHA and IL-2 (40U/ml) starting from the 3 day; the remaining part was made to adhere on the beads bearing the anti-CD3/28 antibodies, thus obtaining a CD8-depleted and CD3/28 positive lymphocytes population. At this fraction IL-2 (40U/ml) was added starting from the 10 day of culture. The cells were counted and this viability was determined each 2-3 days. The ratio beads:cells was maintained constant. The number of cells during time is reported.

FIG. 9. Antiviral effect of co-stimulation with anti-CD3/28 beads on PBMC of *Macaca fascicularis*. The CD8-depleted and CD8-deplete lymphocytes CD3+/CD28+, obtained with the methods described in fig. 8 from 4 monkeys, were stimulated as described in example 5. The two fractions were infected *in vitro* at the 0 day with 0.1 M.O.I. of SIVmac251/63M. The stimulation was performed with PHA and IL-2 added from day 3, and with the anti-CD3/28 beads without addition of exogenous IL-2. The viral production was evaluated by determining the p27 levels (ng/ml) on the supernatants of the culture at day 6 and 12 from infection as described in example 5.

Example 1. Expression, purification and characterisation of the Tat protein (HIV-1 insulated), mutants of the Tat protein and wild type peptides and mutants of Tat.

Many difficulties have been encountered in the past to purify and maintain the biological activity of the Tat protein owing to the easiness of its oxidation, aggregation and loss of activity This is due to the high amount of cysteine, which form intra- and inter-molecular bonds thus modifying the conformation of the native protein [Viscidi et al., Science 246:1606 (1989); Ensoli et al., J. Virol. 67:277 (1993)]. The cDNA or the tat gene (Seq. 1, example 2), which has been cloned in the pL-syn vector, furnished by Dr. J. F. DeLamarter and B. Allet (Glaxo Institute for Molecular Biology S.A., Ginevra, Svizzera), has been used for the expression of the protein in E.Coli.

A first method which we used and which lead to obtain an active protein, was based on successive steps of high pressure liquid chromatography liquid and ion-exchange chromatography [Bohan et al., Gene Expr. 2:391 (1992); Ensoli et al., J. Virol. 67:277 (1993)]. The thus obtained protein is pure at more than 95% and it is active [Ensoli et al., J. Virol. 67:277 (1993); Ensoli et al., Nature 371:674 (1994)], however a good reproducibility was not obtained from batch to batch, owing to the protein oxidation, which is the main problem in commercial Tat preparations. Owing to our observations that Tat basic region has a strong affinity for eparine and that eparine prevents its oxidation, we used the affinity chromatography with heparin and defined a new Tat purification protocol, as described by Chang et al., [AIDS 11: 1421 (1997)]. Cells (10 gr. in weight) of *E.coli* expressing Tat were sonicated in 40 ml of lysis buffer (disodium phosphate 20mM, pH 7.8; glycerol at 2.5%; PMSF 0.2 mM;

DTT 5 mM; mannitol 50 mM; ascorbic acid 10 mM; NaCl 500 mM) by using an Ultrasonic Liquid Processor (Model XL2020, Heat System Inc) with three discharges, each of 20 sec.. The lysate was centrifuged at 12,000 g for 30 min. and the supernatant was incubated for one hour at room temperature with 2 ml of resin heparin sepharose, pre-washed with the lysis buffer. The resin was charged on a glass column and washed with the lysis buffer till the protein was no more detected in the washing medium. The bonded material legato was eluted with lysis buffer containing 2M NaCl and the eluate was collected in fractions of 1 ml. The homogeneity of the eluted protein was analysed by gel electrophoresis (SDS-PAGE). The purified protein was stored lyophilised at -70°C and resuspended in buffer degassed before use.

The biological activity of purified Tat protein, according to the above described protocol, was evaluated with an assay of "rescue" of the viral infection in HLM-1 cells, derived from HeLa-CD4+ cells containing defective proviruses in the tat gene, obtained and described by Sadaie et al. [New Biol. 2:479 (1990)]. The "rescue" assay for the viral infection, described by Ensoli et al. [J. Virol. 67:277 (1993)], consisted in complementing the lack of Tat expression in HLM-1 cells (2×10^5) by means of the addition of exogenous Tat protein (2 μg/ml) and evaluating the viral replication by means of the determination, with an ELISA kit, of commercial "antigen capture", of the concentration of p24 antigen released in the culture medium 48 after the addition of the exogenous Tat protein. The results of the "rescue" expressing, described by Chang et al. [AIDS 11: 1421 (1997)], demonstrate that the Tat protein, purified with this method was active and that with this method the purification was better, easier and less expensive both for purity and for biological activity with

respect to the previously described method [Ensoli et al., Nature 345:84 (1990), J. Virol. 67:277 (1993), Nature 371:674 (1994)].

Different preparations of recombinant Tat, purified as described in the above were inoculated in presence of Freund adjuvant in mice and rabbits, according to standard protocols [(Antibodies - A laboratory manual, Eds. Harlow E., Lane D., Cold Spring Harbor Laboratory (1988)]. The results of the antibody response induced by the immunisation are shown in Table 1.

TABLE 1.

Analysis of the specific anti-Tat antibody response in sera of mice and rabbits immunised with the recombinant Tat protein[a].

anti-Tat antibody	OD-ELISA/Tat			Western blot
	1:500	1:1000	1:2000	
rabbit	0.651	0.400	0.175	+
rat	0.502	0.240	0.150	+

[a] *The recombinant Tat protein produced in E.coli was utilised to immunise mice and rabbits according to standard immunisation protocols [(Antibodies - A laboratory manual, Eds. Harlow E., Lane D., Cold Spring Harbor Laboratory (1988)]. The sera of the immunised animals were analysed by ELISA assay for the presence of anti-Tat antibodies by using three serum dilutions (1:500 at 1:2000). The results are the mean of the readings at 405 nm of two rabbits and three mice. Moreover the sera were tested by Western blot for the recombinant Tat protein (100 ng) migrated in gel SDS-polyacrilamide.*

The results of Table 1 demonstrate that the recombinant Tat prepared by us was able to induce an antibody response in both animal species, as tested with ELISA

and Western blot, by utilising the recombinant Tat protein as antigen. Such antibodies were able to inhibit the internalisation and the biological activities of Tat [Ensoli et al., Nature 345:84 (1990), J. Virol. 67:277 (1993), Nature 371:674 (1994)]. The pL-syn vector and the purification protocol of Tat protein are used to express and purify the mutants of Tat described in Example 2. The biological activity of the mutated and purified Tat proteins is measured by "rescue" tests for the viral infection in HLM-1 cells, assays of cellular proliferation KS cells and in vivo in mice, as described in the above for the wild-type Tat protein. Moreover, the mutated Tat proteins are tested in presence of wild-type Tat (at serial concentrations) to verify the negative transdominant effect on the viral replication in "rescue" tests in HLM-1 cells. The pL-syn vector and the purification protocol are used to express and purify fusion proteins of this type: Tat (wild type or mutants thereof)/IL-12 or Tat (wild type or mutants thereof)/IL-15 or parts of the same or Tat (wild type or mutants thereof)/other molecules (or parts thereof) able to enhance the immune response to Tat alone or associated with other viral products. Fusion recombinant molecules are bilt utilising the sequences and the primers described in examples 2 and 3. As an alternative, as immunogens synthetic peptides are utilised corresponding to regions of Tat or other viral products or of cytokines to be used in combination with Tat. The peptide sequences of Tat are:

Pep. 1. MEPVDPRLEPWKHPGSQPKT

Pep. 2. ACTNCYCKKCCFHCQVCFIT

Pep. 3. QVCFITKALGISYGRK

Pep. 4. SYGRKKRRQRRRPPQ

Pep. 5. RPPQGSQTHQVSLSKQ

Pep. 6. HQVSLSKQPTSQSRGD

Pep. 7. PTSQSRGDPTGPKE

The Tat mutant peptides will contain the same amino acid substitutions of mutated Tat proteins described in Example 2. The peptides will be utilised in combination with the peptide representing the universal T-helper epitope of the tetanic toxoid or of other T-helper peptides [Lanzavecchia, Science 260: 937 (1993)].

Example 2. Construction and characterisation of tat gene mutants

We produced 19 mutants in different Tat regions by means of site specific mutagenesis or of deletion. The sequence of each mutated DNA was controlled by means of sequencing. The tat gene mutated cDNAs were cloned in the PstI site of the pCV0 vector, described in Example 3. Each mutant was co-transfected, as described by Ensoli et al. [J. Virol. 67:277 (1993)], in COS-1 cells, or in the Jurkat T lymphocytes line, with the HIV-1 LTR-CAT plasmide, in which the Cat reporter gene is placed under the transcriptional control of the HIV-1 LTR. The results of those experiments, not published are reported in Table 2.

TABLE 2.

Effects of Tat mutants on the HIV-1 LTR-CAT transactivation and block effect (negative transdominant) on the Tat wild-type activity

MUTANTS	Transactivating activity [a]		Transdominant activity[b] (% inhibition)
	Mean (fold)	(min-max values)	Mean
CYS 22	0.09	(.021-.22)	21
THR 23	0.36	(.16-1)	
THR 23A	0.30	(.16-.78)	
ASN 24	0.34	(.34-.82)	
ASN 24A	0.42	(.45-.95)	
TYR 26	0.14	(.08-.19)	
LYS 28/29	0.52	(.19-1.04)	
CYS 30	0.30	(.045-.65)	
CYS 31	0.60	(.27-1.09)	
PHE 32	0.31	(.077-.097)	
LYS 33	0.04	(.0027-.068)	46
GLU 35	0.31	(.19-.43)	
PHE 38	0.05	(.043-.057)	98
LYS 41	0.04	(.025-.061)	97
TYR 47	0.58	(.31-.8)	
57 A	0.35	(.26-.44)	
TAT-RGD	0.94	(.73-1.15)	
TAT-KGE	1.11	(.67-1.49)	
TAT wild-type	1	1	

a The results are given as activation increments relevant to CAT activity values induced by the wild-type Tat (Fold=1). b The results are expressed in inhibition percent (%) of the wild-type Tat activity.

From the results presented in Table 2 it can be observed that for the majority of the mutants the transactivating effect of the HIV-1 LTR was very reduced or absent, with the exception of the deletion mutant of the RGD sequence, which had an activity similar to the activity of the wild-type Tat. We selected the 4 mutants (cys22, lys33, phe38, lys41) having the lowest (almost zero) transactivating activity and we determined the negative transdominating effect on the transactivating activity of wild-type Tat. To this end COS-1cells were co-transfected with each vector containing a Tat mutant and the pCV-Tat vector (in a moral ratio of 10:1) in the presence of the HIV-1 LTR-CAT vector. as can be seen in Table 2, the lys41 and tyr47 mutants inhibited almost completely the Tat activity, while the lys33 and cys22 mutant partially inhibited the Tat activity. However, the cys22 recombinant protein (described in following Example 3) was able to compete with the wild-type Tat protein in transactivating the HIV-1 LTR-CAT (fig. 1). A mutant in the cysteine region (cys22), one in the core region (lys41), one in the second deleted exon of Tat of the RGD (RGDΔ) sequence and a double mutant containing the mutation in lys41 and the deletion of the RGD sequence (lys41-RGDΔ) were selected.

The sequence of the tat insert and of the mutants selected for the vaccination is reported hereinafter. A series of tat gene mutants is described prepared trough 1) substitution of a base to obtain an amino acidic substitution and 2) deletion of a base sequence to obtain a deletion of the correspondent amino acids. The bases substitutions and deletions were obtained by site direct mutagenesis. The sequences of the wild-type tat gene and of the tat gene mutants, hereinafter reported, were inserted in the pCV0 plasmidic vector as described in the above.

With Seq. 1 it is intended the HIV-1 tat gene sequence, BH-10 clone and [illegible]

derived protein. With Seq. 2 it is intended the cys22 mutant sequence (and of its derived protein), represented by a substitution of Timine (T) nucleotide in position 66 starting from the 5' end with the Guanine (G) nucleotide. This is a substitution in the derived amino acidic sequence of one Cysteine (C in one letter code) in position 22 at the amino-terminal end, with a Glycine (G in one letter code). With Seq. 3 it is intended the lys41 mutant sequence (and of its derived protein), represented by a substitution of the Timine (T) nucleotide in position 123 from the 5' end with the Cytosine (C) nucleotide. This is a substitution in the derived amino acidic sequence of a Lysine (K in one letter code) in position 41 from the amino-terminal end, with a Threonine (T in one letter code). With Seq. 4 it is intended a sequence of the RGD mutant (and of its derived protein), represented by the deletion of the nucleotide sequence CGAGGGGAC, from nucleotide 232 to nucleotide 240, starting from the 5' end of the wild-type tat gene. In the derived amino acidic sequence those is a deletion of the amino acids Arginine-Glycine-Aspartic acid (RGD in one letter code) in the positions 78-80 from the amino-terminal end. With Seq. 5 it is intended a sequence of the double lys41-RGDΔ mutant (and of its derived protein), originated by the combination of the above described mutants.

Wild-type tat nucleotide sequence (Seq. 1)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGC
CTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT
TGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAG
CGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAG
CCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAGGAATAG 3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALG

ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Cys22 mutant nucleotide sequence (Seq. 2)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGC

CTAAAACTGCGGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT

TGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAG

CGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAG

CCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAGGAATAG3

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTAGTNCYCKKCCFHCQVCFITKA

LGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-CaOH

Lys41 nucleotide sequence (Seq. 3)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGC

CTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT

TGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAG

CGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAG

CCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTALG

ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

RGDΔ mutant nucleotide sequence (Seq. 4)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGC

CTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT
TGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAG
CGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAG
CCCACCTCCCAATCCCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Lys41-RGDΔ mutant nucleotide sequence (Seq. 5)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGC
CTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT
TGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAG
CGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAG
CCCACCTCCCAATCCCCGACAGGCCCGAAGGAATAG3'

Amino acidic sequence

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTALG
ISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Example 3. Construction and characterisation of the DNA immunogens.

The DNA molecules for the inoculation of animals are built in the pCV0 plasmide vector of 6.4 kb [Arya et al., Science 229:69 (1985)]. This plasmide comprises two replication origins of SV40, the major late promoter of the adenovirus (AdMLP), and the splicing sequences of the adenovirus and of the mice immunoglobulines genes, the cDNA of mice dihydrofolate-reductase gene (dhfr) and the SV40 polyadenilation signal. The site for the restriction enzyme PstI is located at 3' of the AdMLP, and represents the site in which the exogenous gene of interest is cloned. The tat gene

cDNA (261 couples of bases) (Seq. 1, example 2) of HIV was derived from the BH10 strain [Ratner et al., Nature 313:277 (1985)] and codifies for a protein of 86 amino acids. The pCV-Tat vector [Arya et al., Science 229:69 (1985)] was obtained by cloning the tat gene cDNA in the pCV0 PstI site, the cDNA gene being thus under transcriptional control of the AdMLP. The choice of this vector is based on that the AdMLP induced a higher expression and release of Tat, with respect to other eucariotic promoters, such as, for instance, the immediate early region promoter of the cytomegalovirus (CMV) as demonstrated by Ensoli et al. [J. Virol. 67:277 (1993)], and reported in Table 3.

TABLE 3.

Expression, subcellular localisation, release and activity of Tat in COS-1 cells transfected with pCV-Tat and CMV-Tat[a].

Vectors	Tat expression			Tat[b] content			Tat activity	
Positive Cells	Nucleus[c] (%)	Cytoplasm[c]		Total	Intracell (%)	Extracell (%)	Intracell[d] (fold)	Extracell.[e] (cpm)
pCV-Tat	5-10	++	++	25	63.5	36.5	50	2,478
CMV-Tat	3-5	++	+	14.6	92.2	7.8	72	2,254
Control	0	-	-	0	0	0	1	1,400

[a]COS-1 cells (5 x 10^6) were transfected by electroporation with 30 μg of pCV-Tat, CMV-Tat or a control DNA. 48 hours after transfection, the Tat expression was evaluated though immunoistochemistry with anti-Tat monoclonal antibodies (given values are the mean of percentage value of positive cells) and through localisation of nuclear and cytoplasmatic Tat. The presence of intra- and extra-cellular Tat was analysed through radioimmunoprecipitation on the cellular extracts (500 μl) and in the culture media (4 ml) and subsequent densitometric lecture (Gelsca

Pharmacia) of the precipitated Tat bands. The activity of intracellular Tat was measured on cellular extracts of COS-1 cells cotransfected with Tat expressing vectors, or the control vector and the LTR-CAT HIV-1 plasmide; the extracellular Tat activity on the AIDS-KS cells proliferation induction (determined by tritiate-timidine incorporation assay) was measured in the culture medium (diluted 1:2 and 1:4) of the cells transfected with plasmides expressing Tat or the control plasmide. The results correspond to the mean of five independent experiments.

[b]Densitometric analysis of the immunoprecipitated Tat protein band. Values are expressed in an arbitrary scale, the total detected minimum value (intra- and extracellular Tat) being 10.

[c]-, negative; +, 50% of Tat-positive cells; ++, 50-100% of Tat-positive cells.

[d]CAT activity after 20 minutes incubation with respect to the control vector, the activation value of which is considered 1.

[e]The AIDS-KS cells growth was measured through a tritiated timidine incorporation assay (standard deviation, DS: 12%). The medium of the cells transfected with control DNA had a tritiated timidine incorporation of 1,400 cpm (DS: 11.5%). The culture medium derived from lymphocytes containing HTLV-II virus (positive control) had a tritiated timidine incorporation of 2,400 cpm (DS: 10%).

From the results of Table 3 it can be observed that in the pCV-Tat transfected cells, compared with the CMV-Tat transfected cells, the Tat-positive cells percentage and the total Tat content are higher, the amount of released Tat is much higher and is related to the total and cytoplasmatic content of Tat, and the biological activity of the extracellular Tat on the AIDS-KS cells growth is in consequence higher. Such results

show that the pCV-Tat vector codifies for a biologically active protein, induces high expression levels of tat gene cDNA and can release from the cells much higher Tat amounts than CMV-Tat vector.

The pCV0 vector is utilised also for the expression of HIV-1 nef, rev and gag genes and of the genes codifying for cytokines IL-12 and IL-15. The cDNAs of nef genes (618 couples of bases, NL43 strain) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995)] and rev (348 couples of bases,. strain NL43) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995)], the gag gene (1500 couples of bases, strain NL43) [Myers et al., Human Retroviruses and AIDS. Theoretical Biology and Biophysics Group. Los Alamos, NH (1995], or the cDNAs of IL-12 genes [Wolf et al., J. Immunol. 146:3074 (1991)] o IL-15 [Grabstein et al., Science 264:965 (1994)] are amplified with the polymerase chain reaction technique (PCR) by using specific primers complementary to the first 15 nucleotides of region 5' (primer forward) (Seq. P1, P3, P5, P7, P9) or to the last 15 nucleotides of region 3' of the gene (primer reverse) (Seq. P2, P4, P6, P8, P10). Moreover, each primer, both forward and reverse, comprises the sequence for the restriction enzyme PstI, thus being able to consent the clonation of the amplified in the pCV0 vector. After the clonation, the inserted genes sequence is controlled by means of DNA sequencing. The pCV0 vector is used also for the Tat co-expression with other viral genes of HIV-1 (rev, nef or gag) or with the genes of IL-12 o IL-15 cytokines. To this end the cDNA of HIV-1 tat gene of 261 couples of bases (Seq. 1, example 2) is amplified through PCR with a primer forward including the sequence for the PstI restriction enzyme (Seq. P11) and a primer reverse complementary to the last 15

nucleotides of tat gene (Seq. P12). The viral genes (nef, rev o gag) or the genes of the IL-12 or IL-15 cytokines are amplified with a primer forward which includes also a sequence of 15 bases complementary to the tat gene 3' region, permitting the gene being in frame with the tat gene (Seq. P13, P14, P15, P16, P17), and a primer reverse including the sequence for the PstI restriction enzyme (Seq. P2, P4, P6, P8, P10). Afterwards, a third PCR reaction is performed in which the DNA template is represented by the amplified of the tat gene and of the gene of interest, the primer forward is represented by the primer utilised in amplifying tat (Seq. P11) and the primer reverse by the one utilised in amplifying the gene of interest (Seq. P2, P4, P6, P8, P10). The amplified tat/gene of interest is purified with agarose gel, digested with PstI and cloned in pCV0. After clonation the sequence of inserted genes in controlled by means of DNA sequencing, while the protein expression is determined by means of transfection as described in the above (Ensoli et al, J. Virol. 67: 2771993).

The sequences of the above mentioned primers are:

Seq. P1. Primer forward Rev: 5'ATGGCAGGAAGAAGC3'

Seq. P2. Primer reverse Rev: 5'CTATTCTTTAGTTCC3'

Seq. P3. Primer forward Nef: 5'ATGGGTGGCAAGTGG3'

Seq. P4. Primer reverse Nef: 5'TCAGCAGTCCTTGTA3'

Seq. P5. Primer forward Gag: 5'ATGGGTGCGAGAGCG3'

Seq. P6. Primer reverse Gag: 5'TTATTGTGACGAGGG3'

Seq. P7. Primer forward IL-12: 5'ATGTGGCCCCCTGGG3'

Seq. P8. Primer reverse IL-12: 5'TTAGGAAGCATTCAG3'

Seq. P9. Primer forward IL-15: 5'ATGAGAATTTCGAAA3'

Seq. P10. Primer reverse IL-15: 5'TCAAGAAGTGTTGAT3'

Seq. P11. Primer forward Tat: 5'ATGGAGCCAGTAGAT3'

Seq. P12. Primer reverse Tat: 5'CTATTCCTTCGGGCC3'

Seq. P13. Primer forward Tat/Rev: 5'GGCCCGAAGGAAATGGCA GGAAGAAGC3'

Seq. P14. Primer forward Tat/Nef: 5' GGCCCGAAGGAAATGGGT GGCAAGTGG3'

Seq. P15. Primer forward Tat/Gag: 5' GGCCCGAAGGAAATGGGTGCG AGAGCG3'

Seq. P16. Primer forward Tat/IL-12: 5' GGCCCGAAGGAAATGTGGC CCCCTGGG3'

Seq. P17. Primer forward Tat/IL-15: 5' GGCCCGAAGGAAATGAGAAT TTCGAAA3'

Example 4. <u>Inoculation in healthy *Macaca fascicularis* of a proteic anti-Tat vaccine: evaluation of safety, tolerability, specific immune response.</u>

The tolerability, the safety and the ability to induce a specific immune response (umoral and cellular) of the recombinant Tat protein produced with the above described method and purified with heparin affinity columns were evaluated in experimental model of non human primates of the cynomolgus monkeys (*Macaca fascicularis*). To activate an ample immune response with a proteic vaccine we used aluminum hydroxide (Alum) which was tested in many models and is the only one approved for experiments in men. Among the particulate adjuvants we used RIBI (of the emulsifying agents group and composed by monophosphorilic A lipid, dimiculate

trealose and skeleton of the bacterial wall of the Calmette-Guerin bacillus) [Audibert et al., Immunol. Today 14:281 (1993); Morein et al., AIDS Res. Hum. Retrov. S10:S109 (1994)].

In the first pilot experiment we inoculated 3 monkeys according to the following scheme:

Recombinant Tat protein (100 μg), resuspended in 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a site. 2). Recombinant Tat protein (10 μg), resuspended in 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a site. 3) Non inoculated control monkey. In days -42 and -35 prior to the first vaccination 10 ml of blood were taken from monkeys to determine the base parameters. Samples of sera and plasma were frozen at -20° or -80°C and utilised lately to resuspend the proteic inoculum. Monkeys 1 and 2 were inoculated at 0 time and after 2, 5, 10, 15, 22 and 27 weeks. In the same days of the immunogen inoculation 10 ml samples of blood were taken to perform the laboratory tests (chemio-clinical analysis, electrolytes, leukocytes, platelets and haemoglobin counts), the evaluation of immunologic parameters, such as the presence of specific immunoglobulines (IgM, IgG, IgA) the Th1 (IL-2, IFNγ) and Th2 (IL-4, IL-10) type cytokines levels, IL-15, the chemokins production (Rantes, MIP-1 and MIP-1), the lymphocyte phenotype (CD4, CD8,CD3, CD14, CD20, CD25, CD56 e HLA-DR, CD45RA and CD45RO), the proliferative response to Tat, the CTL and NK activity presence, and the CD8+ (CAF) cells mediated antiviral activity presence. Moreover, to evaluate the in vivo presence of a cell-mediated response, all the monkeys were vaccinated and the control ones were subjected to skin-test for Tat. This pilot experiment includes a further inoculation of all the monkeys at week 32 and a last

inoculation with Tat protein resuspended in Iscom (immune stimulating complex). Iscom is an adjuvant composed by saponine Quil A, cholesterol and phospholipides and able to increase the umoral and cellular response [Morein et al., AIDS Res. Hum. Retrov. 10:S109 (1994); Lövgren et al., Vaccine 14:753 (1996)]. The protective effect of vaccination will be measured after the challenge of monkeys, vaccinated and control, performed at week 41 from the beginning of the immunisation, intravenously inoculating 10 MID_{50} (50% monkey infectious doses) of simian-human immunodeficiency virus (SHIV) developed and titred in *Macaca fascicularis* and containing the tat gene and/or the nef and rev gene of HIV [Shibata et al., J. Virol.65:314 (1991); Li et al., J. AIDS 5:639 (1992); Sakuragi et al., J. Gen. Virol. 73:2983 (1992); Li et al., J. AIDS 5:639 (1992); Igarashi et al., AIDS Res. Hum. Retrov. 10:1021 (1994); Luciw et al., Proc. Natl. Acad. Sci. 92:7490 (1995); Reinmann et al., J. Virol. 31: 98 (1996)]. The post-challenge monitoring (every other week starting from the challenge day for the first month, every 4 weeks for the subsequent 3 months, and every 8 weeks up to 6 months from the challenge) will comprise also the analysis of viral parameters such as determination of the p27 plasmatic values and the viral amount in plasma and in cells. The results of this experiment relating to the first 27 weeks from the beginning of the protocol are as follows. In the vaccinated and control monkeys were not observed significant alterations of physico-chemical, haematological and behavioural parameters. No inflammation or vascular neoformation traces were observed in the inoculation sites. Those results show that the Tat protein was well tolerated by the animals and was non toxic in the administrated doses, utilising the selected inoculation route. In monkeys 1 and 2 specific anti-Tat IgG antibodies were present starting from the 5th

week after the first inoculation. At week 27 anti-Tat IgG were detectable at dilution 1:3200 and 1:6400 in the plasma of monkeys 1 and 2 respectively. The results are reported in figures 2 and 3. The results available at the moment show the onset of a proliferative response to Tat at week 22 (Table 4) in monkeys inoculated with Tat, the response being higher in monkey 2 which received at each inoculation 10 μg of recombinant Tat protein.

TABLE 4

Proliferative response to Tat at week 22 post-immunisation[a]

Monkey N¡	Stimulus	Stimulation index
1	PHA	13.95
	TT	4.68
	Tat	2.45
2	PHA	11.57
	TT	3.77
	Tat	3.04
3	PHA	19.9
	TT	6.23
	Tat	1.38

[a]*Peripheral blood lymphocytes insulated through Ficoll gradient and inoculated at 2x10^5 cells for well in triplicate in 96 well plates; were growth in RPMI 1640 containing 10% of fetal calf serum and stimulated with Tat (5 μg/ml). PHA (4 μg/ml), or tetanic toxoid (TT) (10 μg/ml), against which all the monkeys were vaccinated.*

Control samples were incubated only with the culture medium. The cellular proliferation increase was determined at fay 5 with tritiated timidine incorporation assay as described [Ensoli et al., IV International Conference on AIDS, Stockholm, 1:241 (1988); Cafaro et al., AIDS Res. Hum. Retrov. 7:204 (1991)]. The results are reported as stimulation index (which was calculated according to the following formula: sample cpm mean/control cpm mean. Values higher than 2) are considered positive. Monkeys 1 and 2 were inoculated with recombinant Tat protein (100 μg or 10 μg, respectively) resuspended n 250 μl of autologous serum and 250 μl of RIBI, and subcutaneously inoculated in a sito. Monkey 3 is a non inoculated control monkey.

The results of this pilot experiment show that the recombinant Tat protein, produced and purified according to the protocol described by us, was non toxic at subcutaneously inoculated doses of 100 and 10 μg and was able to induce a specific both umoral and cell-mediated immune response. The anti-Tat specific immune response was higher in monkey 2, immunised with 10 μg of recombinant protein. Moreover, also the RIBI adjuvant was apparently non toxic in animals.

In a subsequently started experiment we evaluated the effect of the immunisation induced by Tat in combination with RIBI or with Alum. The monkeys were subcutaneously inoculated in a single site according the following route. Monkeys 1-3 : 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of RIBI. Monkeys 4-6: 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of Alum. Monkey 7: RIBI 250 μl and 250 μl of autologous serum. Monkey 8: Alum 250 μl and 250 μl of autologous serum. Four of

the monkeys 10 ml of blood were taken at day -9, before the first vaccination, in orther to carry out the analysis described in the first pilot experiment and to have the base parameters of each animal. The monkeys were inoculated at 0 time and after 2, 6, 11 and 15 weeks. In the same days of the immunogen inoculation 10 ml of blood were taken to carry out the tests described in the first pilot experiment. Moreover, urine samples and vaginal tampons were taken on which the presence of specific secretory IgA will be determined. This pilot experiment involves two boosters at weeks 21 and 26 for monkeys 1-6 with the proteic immunogen and adjuvants and for monkeys 7 and 8 only with adjuvants. Finally, it will be performed a last booster at week 31 with the Tat protein Tat resuspended in Iscom. The protective effect of the vaccination will be therefore determined after the challenge of the vaccinated and control monkeys, which will be performed at week 40 from the beginning of the vaccination, by intravenously inoculating 10 MID_{50} of SHIV. The post-challenge monitoring will be carried out as described for the first pilot experiment.

The results of the second pilot experiment, available at present and relating to week 15 from the beginning of the vaccination, are as follows. In the animals there are not observed significant alterations of physico-chemical, haematological and behavioural parameters. The monkeys did not present inflammation or neo-vascularisation signs in the inoculation sites. It has been demonstrated the presence of a specific antibody response (IgM, IgG). At week 15 the anti-Tat antibodies titres (IgG) reached high values which varied from 1:12.800 to titres higher than 1:50.000 (figures 4-6). Moreover, the neutralising activity for Tat in the monkeys sera was tested by inhibition assays of the rescue of the viral infection in HLM-1 cells treated with the exogenous Tat protein, as described in the above [Ensoli et al., J. Virol.

(1993)]. Such tests demonstrate that the monkeys M1-6 sera, diluted 1:2, obtained at week 15 from the first inoculum, were able to block the viral replication induced by exogenous Tat, measured by determining the p24 antigen in the supernatant of the cells, while the serum of the same monkeys at 0 time (pre-immune) or the negative controls did not show activities blocked towards Tat (Table 5).

TABLE 5

Neutralising activity of anti-Tat antibodies on the rescue of the viral infection induced by extracellular Tat.

Samples	HIV-1p24 (pg/ml)
Tat	61.88
Tat + Pre-immune serum M1	46.80
Tat + Pre-immune serum M2	22.96
Tat + Pre-immune serum M3	96.98
Tat + Pre-immune serum M4	126.68
Tat + Pre-immune serum M5	27.03
Tat + Pre-immune serum M6	78.61
Tat + Immune serum M1	4.77
Tat + Immune serum M2	4.88
Tat + Immune serum M3	Neg.
Tat + Immune serum M4	Neg.
Tat + Immune serum M5	7.88
Tat + Immune serum M6	4.55
Pool M1-6 (pre-immune control)	Neg.
Pool M1-6 (immune control)	Neg.
PBS + 0.1% BSA	Neg.

The neutralising activity of the anti-Tat antibodies was determined in HLM-1 cells

(HeLa cells containing an integrated copy of the defective for tat gene HIV-1 provirus). HLM-1 cells were plated at a concentration of 6 x 10^5/plate in 24 wells plates and incubated at 37°C for 16 h. The cells were twice washed with PBS containing bovine serum albumin (BSA) at 0.1%, and incubated for 48 h on culture medium (0.3 ml) to which recombinant Tat protein had been added (30 ng/ml) both alone or in presence of a same volume of monkeys sera (diluted 1:2) taken at 0 time (pre-immune sera) or at week 15 (immune sera). The controls were the cells only treated with a pool of the pre-immune, immune sera, or with PBS containing BSA at 0.1% (PBS + 0.1% BSA) but without Tat. Each sample was double tested. Afterwards, the presence of the cells released virus was tested on the cells supernatants by testing the p24 antigen values, by utilising a p24 antigen capture - Elisa (Nen Dupont) commercial kit. The results are expressed as values of p24 (pg/ml) present in the media and correspond to the mean of the values obtained from two wells for each sample.

At week 11 it was also evident a proliferative response, specific for Tat in monkeys 2 and 3 inoculated with Tat protein and RIBI, and monkey 4 inoculated with Tat protein and Alum, which increased at week 15 (Table 6).

TABLE 6.

Proliferative response to Tat[a]

		Weeks from the beginning of the immunisation			
Monkey N°	Stimulus	0	6	11	15
1	PHA	16.96	22,83	10.50	15,27
	TT	11.69	2,16	1,96	3,01
	Tat	1.12	1.94	1.55	0,52

2	PHA	31.27	29.37	25.75	21,28
	TT	1.12	2.16	1,8	0,57
	Tat	1.08	2.05	3.65	6,22
3	PHA	22.42	39.15	7.89	16,88
	TT	11.43	1.72	0.95	1,71
	Tat	1.65	1.44	2.69	18,82
4	PHA	3.88	13.85	20.77	15,22
	TT	2.85	3.90	4.49	9.07
	Tat	1.29	1.88	3.01	3,24
5	PHA	6.50	11.39	5.74	16,74
	TT	2.31	3.22	1.07	4.84
	Tat	1.80	1.02	0.66	1.76
6	PHA	11.96	7.01	17.94	2,77
	TT	4.14	5.01	1.71	0.13
	Tat	1.37	2.47	1.06	0.11
7	PHA	21.65	25.20	20.30	37,93
	TT	0.97	1.30	0.80	0,88
	Tat	1.78	1.12	0.68	0.73
8	PHA	26.51	21.44	67.09	16,38
	TT	1.20	2.03	10.78	0.20
	Tat	1,12	0.97	0.00	0,21

[a]*Insulated, plated and grown as described peripheral blood lymphocytes were stimulated with PHA (4 μg/ml), the tetanic toxoid (TT) and Tat (5 o 1 μg/ml) and tested as described in Table 4. Monkeys 1-3 were inoculated with 10 μg of recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of RIBI; 3 Monkeys 4-6 were inoculated or with 10 μg recombinant Tat protein resuspended in 250 μl of autologous serum and 250 μl of Alum; two control monkeys*

were inoculated with RIBI (250 µl and 250 µl of autologous serum)(monkey 7) and with Alum (250 µl and 250 µl of autologous serum)(monkey 8).

Moreover, at week 15 five monkeys inoculated with the recombinant protein (monkeys 2-6) reacted to the skin test, further indicating the presence of a cell-mediated response enhanced by a strong retarded hypersensitivity reaction (Table 7 and figure 7).

TABLE 7.

Skin-test to Tat[a]

	Weeks from the beginning of the immunisation	
Monkey N°	11	15
1	-	-
2	-	+
3	-	+
4	-	+
5	+	+
6	-	+
7	ND	ND
8	ND	ND

[a]*Tat (5, 1 and 0.2 µg) in 150 µl of PBS-0.1% BSA or its buffer were intradermally inoculated in a shaved area on the back of the vaccined animals, with the exclusion of the controls (ND, not determined) at week 11 and 15 after the first vaccine inoculation. The onset of an erythema after 24, 48 or 72 hours was considered a positive response.*

Such results show that the immunogen, and also the adjuvants RIBI and alum, were well tolerated by the animals and were non toxic, confirming the results referring to safety and tolerability of Tat vaccination obtained in the first pilot experiment. Moreover, such data confirm the finding reported in the first experiment that the Tat recombinant protein induces a strong specific anti-Tat umoral and cellular in vitro and in vivo.

Example 5. Co-stimulation with anti-CD3/28 beads of CD4+ lymphocytes purified from SIV+ infected monkeys induces the logarithmic expansion of the cells number in absence of significant virus replication and propagation.

The peripheral blood cells were depleted of the CD8 sub-population with anti-CD8 paramagnetic beads (Dynal, Oslo; Dynabeads M-450 CD8) and the purity of the obtained populations was evaluated through cytofluorimetric analysis and considered acceptable if higher than 95%. The depleted CD8 cells (called $CD8^-$ PBMC) were cultivated and stimulated with PHA (2µg/ml) and IL-2 (40 U/ml) or with paramagnetic beads previously conjugated with two monoclonal antibodies specific for antigens CD3 (Clone FN18, Biosource) and CD28 (Clone 9.3, courtesy of Dr. Carl June) (anti-CD3/28 beads). To facilitate the bonding between anti-CD3/28 beads, the incubation occurred on a rotating device and the conjugated cells, selected with a magnet, were called CD8-CD3+CD28+ and cultivated. The cells concentration was brought back to the starting one three times per week and IL-2 was added where indicated; moreover, concerning the cells stimulated with anti-CD3/28 beads, preliminary experiments did show the convenience of utilising a continuous stimulation regimen, restoring the optimal beads/cells ratio at each counting. Our studies did show that in absence of exogenous IL-2 the CD8

CD3+CD28+ sub-population grows significantly better than the $CD8^-$PBMC cells, in response to the co-stimulation with anti-CD3/28 beads and that the addition of IL-2 (40 U/mL, 3 three times per week) significantly enhances the proliferation kinetics referring both to duration and to obtained cells number (figure 8).

To evaluate the antiviral activity, CD8-CD3+CD28+ cells purified from 4 SIV^- animals were infected at day 0 with 0.1 M.O.I. of SIV and maintained in continuous stimulation; as control $CD8^-$PBMC were used, stimulated with PHA and IL-2. The course of the infection was monitored by p27gag viral antigen determination in culture supernatants utilising a commercial ELISA kit (Coulter, Hialeah, FL). The p27 levels (ng/ml) measured at day 6 and 12 after infection and reported in Fig. 9 did show a sharp difference between the two stimulation condition in terms of infection productivity. In fact at day 6 the p27 percent reduction with reference to samples stimulated with PHA and IL-2 was comprised between 40% and 87% and at day 12 such reduction was enhanced in 2 out of 4 samples suggesting a lesser cells permissivity to the viral infection. Only in a case (mk 9401) we registered the infection propagation even with anti-CD3/28 beads stimulation. The described results show that *Macacus fascicularis* is a good model for the ex vivo expansion of lymphocitary sub-population in absence of significant viral replication through co-stimulation with anti-CD3/28 beads and is the basis for a therapeutic vaccine based on expansion and re-infusion, in HIV infected subjects, of autologous lymphocytes specific for the proposed viral antigens.

Prophetic example 6. Inoculation in *Maraca fascicularis* of a anti-Tat DNA vaccine.

The direct inoculation is proposed of pCV-Tat plasmide DNA, containing the cDNA of wild-type tat gene, and of pCV0 as control DNA. The plasmidic DNA to be administered to the animals are amplified in E.Coli bacteria (strain DH5) according to standard procedures [Molecular cloning - A Laboratory manual; Eds. Maniatis T., Fritsch E.F., Sambrook J., Cold Spring Harbor Laboratory, Cold Spring Harbor, New york (1992)] and according to protocols established by "European Agency for the evaluation of medicinal products; Human Medicine Evaluation Unit" (Technical Report Series No. 17 January 1997), purified by means of two CsCl gradients and dialysed for 48-72 hours against 100 volumes of sterile PBS (without Ca++ and Mg++). The DNAs are controlled through digestion with restriction enzymes, whose cut sites on plasmidic DNA are known, and their functionality is controlled through transfection of 5-10 μg of DNA with the calcium phosphate technique [Molecular cloning - A Laboratory Manual; Eds. Maniatis t., Fritsch E.F., Sambrook J., Cold Spring Harbor Laboratory, Cold Spring Harbor, New york (1992)] in H3T1 (1×10^6) cells, deriving from HeLa cells containing, integrated, a copy of the plasmide reporter HIV-1 LTR-CAT, and 48 hours after determination of the CAT enzyme [Gorman et al. Mol. Cell. Biol. 2:1044 (1982)].

The pCV-Tat or pCV0 DNAs (0.5-2 mg) resuspended in physiologic solution will be intramuscularly inoculated in two sites. 2-5 days before vaccination the animals will be inoculated with 1 ml of physiological solution containing bupivacaine 0.5% and metilparabene 0.1% in the two duly marked sited, in which later on the vaccination will be made, to increase the uptake and the DNA expression in the muscle [Danko et al., Vaccine 12:1499 (1994); Fine et al., Ann. Plast: Surg. 20:6 (1988)]. The monkeys will be inoculated at time 0 and after 5, 10, 15, 22 and 27 weeks

32 a booster will be done with the recombinant Tat protein (10 μg) in presence of Iscom. The animals will be daily monitored as regards to the clinical parameters as described in example 4. Moreover, before the immunisation and in the same days of the inoculation, as described in example 4, 10 ml blood samples will be taken. The protecting effect of the vaccination will be measured after the monkey challenge, which will be diminished at week 41 after beginning of the immunisation, intravenously inoculating 10 MID50 of SHIV. The post-challenge monitoring will be performed as described in example 4. Immunisation protocols will be also evaluated, in which there will be included combinations od DNAs expressing the other HIV-1 genes and/or cytokines, described in example 3.

The pCV0 and pCVTat plasmidic DNAs can also be inoculated in the animals utilising other delivery systems, which could make stronger the immunisation, such as the use of liposomes, nanoparticles or gene gun.

Prophetic example 7. Use of dendritic cells

The dendritic cells precursors will be isolated from peripheral blood of non-human primates of the Macaca *fascicularis* species through in vitro culture of adhering cells with GM-CSF and IL-4 for 7-14 days. The morphological analysis and the phenotypic characterisation (FACS analysis and immunoistochemistry after cytospin) will be made to confirm the identity of the cells obtanied in the culture. The functional analysis will be based on the capacity of the isolated cells to induce the proliferation of allogenic lymphocytes, peculiar characteristic of dendritic cells.

The dendritic cells resuspended at the $1x10^5$/100 μl concentration in RPMI 1640 containing 5% of autologous serum, 10 mM of Hepes buffer, 100 U/ml of penicilline-streptomicine, 0.5 mg/ml of amfotericine B, and glutamine at 0.03%, will be

incubated for 2 hours at 37°C in presence of Tat or of its peptides or of the combinations Tat, Rev, Nef, Gag and/or cytokines and successively intravenously inoculated 2 or more times at intervals of 2-4 weeks. As an alternative, the dendritic cells will be transduced with vectors containing the tat gene alone or in combination with the other already mentioned vectors and subsequently intravenously inoculated.

Prophetic example 8. Therapeutic vaccination.

To define the efficiency of an anti-Tat therapeutic vaccination, both proteic and DNA, experiments will be made in monkeys already infected with SHIV in the asymptomatic and symptomatic phases. In particular, the onset of alteration in the inoculation site and of general symptoms in the asymptomatic monkeys, and of modification of the symptomatology in already symptomatic animals will be evaluated. In fact, an even remote probability exists that in infected monkeys the Tat inoculation will increase the viral replication, however such an effect should be a transitory one, considering both the low Tat inoculated dose and the short half-life of the transactivating protein (T1/2: 12 h). Should be the case, the Tat mutants will be used (in form of proteins peptides or DNAs), described in example 2. As an alternative, we will use in combination with the vaccine, inhibitors of the viral replication. To evaluate the vaccination effects on the illness progression, the monitoring of clinical, laboratory and viro-immunological parameters (above described) will be made at 0 time, at the moment of the next administration of the immunogen and 30 days after the same. After the last inoculation will take place monthly and each time a sudden change of the clinical situation will appear. PBMC, serum, plasma and urine samples will be frozen at each control for further evaluation as described before.

Prophetic example 9. Stimulation of the mucosal immunity

The described immunogens will be utilised to induce and/or enhance a specific immune response at mucose level. One of the routes will be used is based on the use of bacteria (Streptococcus gordonii, host in the oral cavity in humans) engineered to express the above mentioned viral antigens. Such bacteria are able to colonise the oral and vaginal mucose in mice and to induce a specific, local and systemic, antibody response, with respect to eterologous antigens expressed on the recombinant bacteria surface [Oggioni et al., Vaccine 13: 775 (1995); Medaglini et al., Proc. Natl. Acad. Sci. USA 92: 6868 (1995); Medaglini et al., Vaccine 1997, in press]. The inventor believes that this method could be successfully utilised to induce also a specific vaginal immunisation in cynomolgus monkeys. As an alternative the mucosal immunity can be induced with the proteic immunogens above described, utilising other also bacterial delivery systems, such as cytofectines and liposomes, and those inoculation routes able to induce the better immune and protective response [Lewis et al., Vaccine Press, Ed. Robinson, Farrar, Wibling; Human Press, Totawa, New Jersey (1996); Lehnen et al., Vaccine Research 1:319 (1992); Honenbang et al., Infect. Immun. 62:15 (1994)].

Claims

1. Vaccine against AIDS tumours and syndromes recurring in HIV-1 infected subjects, comprising recombinant protein or peptides of the wild-type Tat and of the mutants thereof (cys22, lys41, RGDdelta and lys41-RGDdelta).
2. Vaccine according to claim 1, wherein the proteins or the peptides and mutants thereof are combined with Nef; Rev or Gag proteins or peptides.
3. Vaccine according to claim 1, wherein the proteins or the peptides and mutants thereof are combined with the IL-12 and IL-15 immunomodulating cytokines.
4. DNA vaccine against AIDS tumours and syndromes recurring in HIV-1 infected subjects, comprising DNA molecules or fragments codifying for wild- type Tat or for the mutants (cys22, lys41, RGDdelta and lys41-RGDdelta), said DAN molecules or fragments being inserted in the pCV0 plasmidic vector
5. Vaccine according to claim 4, wherein the DNA molecules or fragments are combined with DNA molecules or fragments of nef, rev and gag genes.
6. Vaccine according to claim 4, wherein the DNA molecules or fragments are combined with DNA molecules or fragments of IL -12 and IL -15 immunomodulating cytokines.
7. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or Nef, Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to immunise autologous dendritic cells induced to express the corresponding antigens.
8. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or

Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to obtain drugs or vaccines for the immunisation of autologous dendritic cells transduced with eucariotic vectors containing said recombinant proteins or wild-type Tat and their mutants and combinations thereof.

9. Use according to claims 7 and 8 to produce drugs or vaccines for mucosal immunisation.

10. Use of recombinant proteins or wild-type Tat and mutants thereof (cys22, lys41, RGDdelta and lys41- RGDdelta), or combinations thereof with proteins or Nef, Rev or Gag peptides, or combinations thereof with IL-12 and IL-15 immunomodulating cytokines to obtain drugs or vaccines for the immunisation of peripheral blood cells through co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies.

Rome,

f. Barbara Ensoli

The Representative

(signature)

Dr. Maria Vittoria Primiceri

of NOTARBARTOLO & GERVASI S.p.A.

MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT

Italian Patent and Trademark Office ROME

****

REQUEST FOR TAKING OVER REPRESENTATION OF A PATENT

The undersigned ISTITUTO SUPERIORE DI SANTIA', of Italian nationality, having seat in ROME, through the Representative Dr. MARIA VITTORIA PRIMICERI (reg. No. 465) et al of NOTARBARTOLO & GERVASI S.p.A., Via Savoia 82, Roma, wherein they have elected domicile in accordance with the law, present owner of application for patent of industrial invention No. RM 97 A 000743 of 01.12.1997 in the name of Ensoli Barbara, but assigned to Istituto Superiore di Sanità with official receipt of assignment recordal (registration No. MIE000907 of 05.08.1998), hereby requests this Ministry to record the taking over the representation of the patent application for industrial invention, in order to carry out any further step concerning the above indicated patent, by the representative NOTARBARTOLO & GERVASI S.p.A., Via Savoia 82, Roma, according to Articles 93 and 94 Royal Decree 29.06.1939 and modified by Decree of the President of Republic 22.6.79 No. 338.

It is enclosed:

- power of attorney.

Milan, date SEAL

f. ISTITUTO SUPERIORE DI SANITA'

The Representative

Dr. MARIA VITTORIA PRIMICERI

NOTARBARTOLO & GERVASI S.p.A.

OMISSIS

MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT

Provincial Office of Industry, Commerce and Handicraft of Milan

Patent Service for Inventions, Models and Trademarks

COPY OF THE FILING RECORD FOR CHANGE OF OWNERSHIP

In the year 1996 n the 5h day of the month of AUGUST

the firm ISTITUTO SUPERIORE DI SANITA'

having place of business in ROME, VIALE REGINA ELENA 299 - ITALY

through representative Dr. DIEGO PALLINI(Reg. No. 484) et al

and domiciled by the election in conformity with the Law in Milan-Corso di Porta Vittoria 9

c/o NOTARBARTOLO & GERVASI S.p.A.

filed before the undersigned:

1) Petition for change of ownership, in duplicate, concerning:

- Patent for { INVENTION No. RM97A000743 OF 01.12.1997
- patent application

2) Power of Attorney

3) Payment receipt on giro account No. 00668004 in the name of Registry of Deeds of ITL 120.000 issued by the Office of on

4) Certified copy of Assignment deed, signed as assignor by Mrs. Barbara Ensoli and signed as assignee by Mr. R.R. Di Giacomo, being both signature authenticated by Dr. G.Floridi, Notary in Rome, on 24.04.1998(Said deed was recorded on 12.05.1998 before the Registrar Office in Rome.

RESERVED TO THE CENTRAL PATENT OFFICE

Deed of ASSIGNMENT recordal No.

From BARBARA ENSOLI RESIDING IN ROME, VIA FESTO AVIENO 176 - ITALY

In favour of ISTITUTO SUPERIORE DI SANITA' HAVING SEAT IN ROME, VIALE REGINA ELENA 299 - ITALY

Patent for INVENTION

Patent application for No. RM97A000743 FILED ON 01.12.1997

The above document has been duly countersigned by me with the office seal.

SEAL

THE DEPOSITOR

(signature)

THE RECORDING OFFICER

(signature)

Copy in conformity with the original

f. THE DIRECTOR

(signature)

"This is to underline that for this application and enclosures, the duty stamp has been paid in conformity with the Circular No. 163/83 of U.C.B. with reserve for possible integration which will be requested by the same on acceptance"

MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT – Italian Patent and Trademark Office – ROME

CHANGE OF OWNERSHIP REQUEST

The undersigned ISTITUTO SUPERIORE DI SANITA', of Italian nationality, having seat in Rome, Viale Regina Elena 299,

through the Representatives: D.ssa Gemma Gervasi (reg. No. 238), Dr. Diego Pallini (reg. No. 484), Dr. Giorgio Moretti (reg. No. 206), Dr. Angelo Passini (reg. No. 73), Ing. Giorgio Coggi (reg. No. 148), Dr. Livio Brighenti (reg. No. 475), D.ssa Maria Vittoria Primiceri (reg. No. 465), D.ssa Raffaella Consuelo Asensio (reg. No. 504), Dr. Giulio Mariani (reg. No. 329), separately, of NOTARBARTOLO & GERVASI S.p.A., Corso di Porta Vittoria 9, Milano

REQUESTS

this Office to record the change of ownership, in favour of the undersigned, of the assignment:

from: BARBARA ENSOLI residing in Rome, Via Fiesto Avieno 176 (ASSIGNOR),

to: ISTITUTO SUPERIORE DI SANITA', of Italian nationality, having seat in Rome, Viale Regina Elena 299, (ASSIGNEE),

regarding the hereinafter mentioned Italian patent:

- Patent Application for industrial invention No. RM97A000743 filed on 01.12.1997 and having title: "HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes" as well as the relevant granted patent;

To this purpose the following documents are attached:

- Certified copy of the assignment deed, signed by the ASSIGNOR personally Mrs. Barbara Ensoli and signed on behalf of the assignee by Mr. Romano Rosario Di Giacomo, being both signatures authentica(by Dr. Giuliano Floridi, Notary in Rome, on 24.04.1998. Said deed was recorded on 12.05.1998 before the Registrar Office in Rome.
- Payment order of Italian Lire 120.000.= in favour of the Giro account No. 00668004 in the name of Reistrar Office of Taxes and Fees – Rome.

Milan, 3 August 1998.

f. ISTITUTO SUPERIORE DI SANITA'

The Representative

(signature)

Dr. Diego Pallini

[Top and RH margin: Stamp of Notary Public, Revenue stamps, Registration stamp]

Reg. No. 24

ISTITUTO SUPERIORE DI SANITA'

DEED OF TRANSFER OF PATENT APPLICATION

BETWEEN

Dr. Barbara Ensoli, born in Latina on January 23, 1960, Italian citizen, resident in Rome, Via Festo Avieno 176, Tax No. NSLBBR60A63E472K, hereinafter referred to, for reasons of brevity, as the TRANSFEROR

on the one hand; and

Istituto Superiore di Sanità, with registered office in Rome, Viale Regina Elena 299, Tax No. 80211730587 and VAT No. 03657731000, in the person of Dr. Romano Rosario Di Giacomo, Director General of Administrative Services of the Institute, born in Cassano Ionio (Province of Cosenza) on September 25, 1937, hereinafter referred to, for reasons of brevity; as the TRANSFEREE

on the other hand;

between the said parties, represented as specified above, it is agreed and stipulated as follows:

Article 1

The TRANSFEROR hands over and transfers to the TRANSFEREE, who accepts, the full and exclusive property, free from any and whatsoever real tie of enjoyment or guarantee, of the Italian patent application specified below (hereinafter referred to as the PATENT):

- PATENT APPLICATION for industrial invention No. RM97A00743 filed on December 1, 1997 and entitled: "Tat of HIV-1 or its derivatives, either alone or in combination, for vaccination, prophylactic and therapeutic use, against AIDS, tumours and associated syndromes".

Article 2

The TRANSFEROR moreover transfers to the TRANSFEREE every right in connection with the filing of patent applications abroad that are based on the European Union priority right referred to in Article 4 of the European Union Convention of Paris, as regards the PATENT referred to in the foregoing Article 1.

Article 3

The PATENT is transferred to the TRANSFEREE in the state of fact and law in which it is found.

The TRANSFEROR foregoes any and every guarantee as regards validity of the PATENT and non-interference of the same with rights of exclusive ownership pertaining to third parties.

Article 4

The TRANSFEREE undertakes to undersign the entire documentation necessary for transcribing the present Deed at the Italian Patent and Trade-mark Office in Rome. Said transcription shall be carried out under the responsibility and at the expense of the TRANSFEREE.

Also payable by the TRANSFEREE are the expenses for copy, stamp, and for registration for tax purposes of the present Deed, as well as the expenses for authentication by a Notary Public of the signatures of the TRANSFEROR and of the legal representative of the TRANSFEREE.

Article 5

The consideration for the PATENT transferred by means of the present deed, entitled "Tat of HIV-1 or its derivatives, either alone or in combination, for vaccination, prophylactic and therapeutic use, against AIDS, tumours and associated syndromes" is agreed at 2 500 000= (two million, five hundred thousand) Italian lira. The above sum shall by paid by the TRANSFEREE by means of payment on the bank account No. 3886 cod. ABI 1005 - C.A.B 03375, made out to Dr. Barbara Ensoli at the Banca Nazionale del Lavoro - window for Istituto Superiore di Sanità Rome.

Article 6

The present Deed shall become binding for the TRANSFEREE only after the approvals and registrations envisaged by law are accomplished.

Read, approved, and signed in two original copies, in the place and on the day specified below:

Rome, April 4, 1998

[Signatures]

= = = =

REPERTORY No. 13436

AUTHENTICATION OF SIGNATURE

THE ITALIAN REPUBLIC

I, the undersigned Dr. Prof. Giuliano FLORIDI, Notary public in Rome, with office in Viale Regina Margherita 239, enrolled in the College of Notaries Public of the United Districts of Rome, Velletri and Civitavecchia, hereby declare and certify true and authentic the signatures of:

- Barbara Ensoli, born in Latina on January 23, 1960 and resident in Rome, Via Festo Avieno No. 176, medical doctor;
- Romano Rosario Di Giacomo, born in Cassano Ionio (Province of Cosenza), on September 25, 1937 and domiciled for the office in Rome, Viale Regina Elena 299, medical doctor;

of whose personal identity I, the Notary Public, am certain, and who have signed the foregoing deed in my presence and after prior express and mutual waiver of the presence of witnesses, with my consent.

Rome, in Viale Regina Margherita 239, on the twenty-fourth day of April, nineteen hundred and ninety-eight.

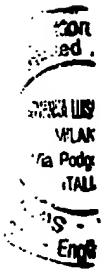
SIGNED BY GIULIANO FLORIDI, NOTARY PUBLIC

= = =

True copy, signed according to the law, which is issued for the uses allowed by law

Rome, June 3, 1998

[Stamp]



MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT

Italian Patent and Trademark Office ROME

****

AMENDMENT AND INSERTION REQUEST

Re: Patent Application for Industrial Invention No. RM97A000743 filed on 01.12.1997 in the name of ISTITUTI SUPERIORE DI SANITA' having title: : "HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes"

According to article 49 of D.P.R. dated June 22, 1979, No. 338, the undersigned Istituto Superiore di Sanità, having seat in Rome, Viale Regina Elena 299, through representatives Dr. Gemma GERVASI (Reg. No. 238) Dr. Diego Pallini (reg. No. 484), Dr. Giorgio Moretti (reg. No. 206), Dr. Angelo Passini (reg. No. 73), Ing. Giorgio Coggi (reg. No. 148), Dr. Livio Brighenti (reg. No. 475), D.ssa Maria Vittoria Primiceri (reg. No. 465), D.ssa Raffaella Consuelo Asensio (reg. No. 504), Dr. Giulio Mariani (reg. No. 329) (separately) of NOTARBARTOLO & GERVASI S.p.A., Via Savoia 82 - 00198 ROME, wherein has elected domicile in accordance with the law,

REQUESTS

this Office to make the amendments and insertions to the specification as hereinafter specified:

Note 1: page 1 of the description: line 16: after the word "antiviral" insert: "in the presence or not of vaccinal adijuvants".

Note 2: page 1 of the description: line 17: after the word "mucosal" insert a comma and erase "or".

Note 3: page 1 of the description: line 19: after the word "anti-CD28" insert: ", and to the delivery of the immunogens using erythrocytes or nanoparticles".

Note 4: page 12 of the description: line 11: after the word "immunomodulant" insert: "such as IFNα or β".

Note 5: page 13 of the description: after line 16: go on a new line and insert: "Recent studies have shown that the co-stimulation of CD4+ lymphocytes with paramagnetic beads, coated with anti-CD3 e anti-CD28 monoclonal antibodies determines a logarithmic and polyclonal expansion of lymphocytes from HIV-infected subjects [Levine et al., Science. 272: 1939-1943 (1996)] without activating virus replication and transmission. Such antiviral activity is a consequence of both the negative modulation of the expression of CCR5, the co-receptor of HIV-1 monocytotropic strains [Carrol et al, Science. 276: 273-276, (1997)] and, to a lesser extent, of the high levels of chemokines induced by the co-stimulation with anti-CD3 and anti-CD28 monoclonal antibodies [Riley et al, J.Immunol. 158: 5545-5553, (1997)]. The inventor believes that the possibility to expand autologous lymphocytes from HIV infected subjects in the absence of viral replication/transmission, permits to obtain an effective ex vivo immunization, described in the examples, which can be highly helpful in developing an anti-Tat vaccine."

Note 6: page 14 of the description: after line 12: go on a new line and insert: "In particular, the inventor believes that the use of (*S. Gordonii* and *Lactobacillus)* bacteria, "modified" to express the above mentioned viral antigens, might be a valid strategy to induce or potentiate a specific immune response at the mucosa level in monkeys and in man. These bacteria are, in fact, able to colonize the mouse oral and vaginal mucosa, and to induce a specific, local and systemic antibody response against heterologous antigens expressed on the surface of recombinant bacteria [Oggioni et al., Vaccine 13:775 (1995); Medaglini et al

roc. Natl. Acad. Sci. USA 92: 6868 (1995); Medaglini et al., Vaccine 15:1330 (1997); Pozzi et al., in "Gram-positive bacteria as vaccine vehicles for mucosal immunization", eds. Pozzi G. & Wells, J.M. – Landes, Austin, p. 35 (1997); Oggioni, et al., Gene 169:85 (1996); Rush, et al., in "Gram-positive bacteria as vaccine vehicles for mucosal immunization", eds. Pozzi G. & Wells, J.M. – Landes, Austin, p. 107 (1997); Medaglini et al., Biotech. Annu. Rev. 3:297 (1997); Medaglini et al., Am. J. Reprod. Immunol. 39:199 (1998)]. Finally, these bacteria act as live vectors and can induce a prolonged stimulation of the immune system. Moreover, the inventor believes that non-replicating and non-pathogenic recombinant viral vectors, such as herpes simplex type-1 viruses (HSV-1) [Marconi et al., Proc. Natl. Acad. Sci 93:11319 (1996)], can be used to express viral proteins for systemic (intradermic) and mucosal (oral, vaginal and nasal routes) immunization. In fact, these vectors can accommodate large exogenous sequences [Glorioso et al., Ann. Rev. Microbiol. 49:675 (1995); Huard et al. Gene Ther. 2:385 (1995)], such as several HIV genes (regulatory, accessory and structural). Moreover, herpes vectors can also be administered via the oral, nasal or vaginal route [Bowen et al., Res. Virol. 143:269 (1992); Kuklin et al., J. Virol. 240:245 (1998)].

The inventor believes that Tat (either as protein or DNA), alone or in combination with the other immunogens described above, can be inoculated also by using new delivery systems, such as erythrocytes or nanoparticles. In particular, the inventor believes that it is possible to deliver antigens bound to the membrane of autologous erythrocytes [Magnani et al., Biotech. Appl. Biochem. 16: 188 (1992) and 20: 335 (1994)]. Since these erythrocytes are removed from the blood by macrophages, professional antigen presenting cells (in men after 120 days), this feature can be used for vaccine purposes. Finally, another delivery strategy is the use of nanoparticles that can carry and release proteins and DNA [Chavany et al., Phar. Res. 9: 441 (1994); Zobel et al., Antisense Nucleic Acid Drug Dev. 7: 483 (1997)] in that they can be produced with a high affinity for such biologically active molecules. Nanospheres are polymeric colloidal particles of diverse chemical composition, variable from 10-1000 nm. Different substances (oligonucleotides,

drugs, proteins, peptides, DNA) can be loaded on their surface or absorbed in the particle and delivered into the cytoplasm or the nucleus of the cells from where they are slowly released. This allows the utilization of very small amounts of the substance to be delivered. Therefore nanoparticles are a most suitable delivery system particularly for those substances which would be naturally unstable in the intracellular environment, or for which effectivness or delivery to specific cells would be enhanced." and erase from line 13 of page 14 to line 2 of page 15 of the description.

Note 7: page 15 of the description: line 5: after the word "parts" insert: "in the presence or not of vaccinal adijuvants".

Note 8: page 15 of the description: line 23: change "Claims" into "Summary of the invention"

Note 9: page 17 of the description: line 19: after "CD28" insert ", in case conjugated to paramagnetic beads".

Note 10: page 17 of the description: after line 21: go on a new line and insert: "11. an anti-Tat vaccine as already described, in combination with adjuvants which increase the immune response.

12. an anti-Tat vaccine, alone or in combination as already described, administered by specific delivery systems, such as nanoparticles, herpes vectors, red blood cells, bacteria or any other delivery system by which the above described vaccine, in all its combinations, can be administered.".

Note 11: page 19 of the description: after line 10: insert "In this regard, for inibitors of viral replication it is intended all molecules known at the present, or those which will be discovered later on (nucleoside and non-nucleoside inhibitors of reverse transcriptase, protease inhibitors, antisense RNA and, in general, all molecules able to block HIV gene expression) able to reduce or block the HIV replication.

As previously said, different methods of immunization are described, which utilize Tat in association with other viral genes or proteins, or part thereof, or immuno modulant cytokines or genes coding for immuno modulant cytokines, or part thereof. For "part thereof" it is intended segments of genes or of proteins, above

escribed, whose efficacy of inducing the same immunogenic effects of the entire gene or protein is demonstrated.

Moreover, since the efficacy of adjuvants in vaccine strategies is known, the present invention refers to the use of known adjuvants and of those which will be discovered later on, administered together with Tat (protein or DNA) and with combinations of Tat and other genes or viral or cellular proteins. Similarly, it is hypothesized the efficacy of different delivery systems of Tat (protein or DNA) and combinations of Tat and other genes or viral or cellular proteins in inducing both a systemic and local immune response to Tat (mucosal immunization).

Results obtained from the inventor (not published), indicate that only the Tat protein, in its biologically active form, is able to bind specific cellular receptors and enter the cell. This characteristic is at the base of the immune response of accessory cells and of the immune cells more in general and, according to the inventor, it is of a fundamental importance in inducing a much stronger immune response than the inactivated protein is able to elicit. In conclusion, unlike the use of inactivated Tat as immunogen, proposed by some scientists, the inventor intends to utilize HIV-1 Tat, or its mutants, in its biologically active form, in order to induce a very strong immune response against HIV, able to prevent infection or the development of the disease and to permit efficient therapeutic strategies in HIV-1-infected individuals. According to the inventor, the vaccine can be delivered through systemic (intramuscular, intradermal, subcute, etc.) or local (mucosal) routes. The last route is preferred when bacteria (see below) are utilized as delivery systems. The vaccine can be produced in lyophilized form. At the moment of use, it can be resuspended in autologous serum.

Transformed cells, comprising a Tat-expressing vector, or Tat mutant-expressing vector, or parts thereof, as previously described, and cells which are cultured to express Tat protein, which will be isolated for the use, are all included in the scope of the present patent.

It is intended that all Tat variants (including all types and subtypes of HIV strains), with analogous or greater activity than that above described, are included in this invention.".

Note 12: page 20 of the description: line 7: change "10 or with 100 µg" into "100(M1) and 10(M2) µg".

Note 13: page 20 of the description: line 9: after the word "inoculated" insert "(M3)" and go on a new line.

Note 14: page 20 of the description: line 10: after "22" erase "and 27 weeks" and insert ", 27, 32, 37 weeks. Antibodies were evaluated also at week 41 in monkey M2, inoculated with 10 µg of Tat protein, and in monkey M3.".

Note 15: page 20 of the description: line 11: change the word "plasma "into "sera".

Note 16: page 20 of the description: line 15: erase "PBSa 1x" and insert "PBS without Ca^{++} and Mg^{++} (PBS-A)".

Note 17: page 21 of the description: line 3: after "1:50" insert "and 1:100".

Note 18: page 21 of the description: line 4: change "(±S.D.)"into "+3 standard deviation (S.D.)".

Note 19: page 21 of the description: line 6: after the word "sample" add: "diluted 1:50".

Note 20: page 21 of the description: line 7: after the word "wells" erase ", subctracted of the cut off value ± S.D. (ΔOD405);" and insert a full stop.

Note 21: page 21 of the description: line 8: after ">2,7" add "indicates that"

Note 22: page 21 of the description: line 10: after the word "Titration" erase "of plasma from monkeys inoculated with 100 and 10 µg recombinant Tat protein, described in Fig. 2." and insert "of anti-Tat antibodies in plasma from monkeys inoculated with 100 (M1) and 10 (M2) µg recombinant Tat protein, described in Fig. 2. ELISA was carried out as described in figure 2. In this case the monkeys plasma were assayed (in duplicate) at scalar dilutions from 1:50 to 1:25.600."

Note 23: page 21 of the description: line 13: after the words "cut off" insert "The cut-off value was calculated for each dilution and corresponded to + 3 S.D. of the readings of the plasma from all monkeys in all experiments at 0 time."

Note 24: page 21 of the description: after line 13: go on a new line and insert "**FIG. 4.** Mapping of the Tat epitopes recognized by the anti-Tat IgG from monkeys injected with 100 (M1) and 10 (M2) µg of recombinant Tat protein, described in

JP006576

Translation priority document.pdf
12/14/07 11:41 AM

fig. 2. For mapping those epitopes recognized by anti-Tat antibodies ELISA were carried out using 8 synthetic peptides corresponding to Tat amino acids (aa) 1-20, 21-40, 36-50, 46-60, 52-72, 56-70, 65-80 and 73-86. 100 µl of each peptide (10 µg/ml in PBS-A/0.1% BSA) were absorbed onto a PVC 96-well plate for 12 hours at 4°C. Plates were then washed and incubated with 100 µl of PBS-A/3% BSA for 2 hours at 37°C. After incubation, plates were washed with PBS-A/0.05% Tween and then 50 µl of plasma, diluted in PBS-A and 3% BSA, were added to each well. Elisa were then continued as described in figure 2. Plasma were obtained at week 37 after the primary immunization. Cut-off values, calculated for each peptide and for each plasma dilution, correspond to the average + 3 S.D. of the readings of plasma at 0 time for all monkeys in all experiments; (A) shows the average results of plasma diluted 1:50 for each peptide tested, corresponding to the optical densities at 405 nm of the readings of two wells; (B) shows the antibody titers of plasma shown in (A), expressed, in ordinate, as the reciprocal of the highest dilution which at 405 nm gave a reading higher than the cut off corresponding to each dilution.".

Note 25: page 21 of the description: line 14: change "4" into "5".

Note 26: page 21 of the description: line 22: after the word "weeks" insert "The search of the antibodies was investigated at 2, 6, 11 and 15 weeks. The ELISA method is described in fig. 2." and erase from "The ELISA" to "fig.2.".

Note 27: page 22 of the description: line 3: after the value "1:1000." add "The cut-off value was calculated as the average (+2 S.D.) of the readings of plasma of all monkeys, taken at 0 time. Results are shown in the histogram as the average of the optical densities at 405 nm of the readings of two wells subtracted of the cut-off value (ΔOD 405).".

Note 28: page 22 of the description: line 4: change: "5" into "6" and after the word "umoral" erase "of" and insert "of the specific anti-Tat" and erase "specific anti-Tat".

Note 29: page 22 of the description: line 5: after "with" erase: "Tat" and add "recombinant Tat protein"

Note 30: page 22 of the description: line 11: after "6, 11" erase:"and 15" and

"lines 12 and 13" and insert ", 15, 21, 28 and 32 weeks. At week 36, monkeys M1 to M6 were inoculated with 15 μg of Tat protein resuspended in 200 μl of ISCO[illegible] and 300 μl of PBS. Antibodies were evaluated also at week 40 and 44. The ELISA method and the cut-off value determination are described in fig. 2. The results shown refer to samples diluted 1:50. >2,7 indicates that the O.D. value was out of scale.".

Note 31: page 22 of the description: line 14: change "6" into "7" and after the word "Titration" erase "of plasma from the monkeys inoculated with" and insert "of anti-Tat antibodies in plasma from the monkeys inoculated the protein".

Note 32: page 22 of the description: line 16: after "(M4-6)" add "described in Figure 6".

Note 33: page 22 of the description: line 18: after the words "cut off" add "calculated for each dilution, as described in Figure 3.".

Note 34: page 22 of the description: after line 18 go on a new line and insert: "**FIG. 8.** Mapping of epitopes of Tat recognized by anti-Tat IgG from monkeys inoculated with recombinant Tat protein (10 μg) in the presence of RIBI (M1-3) or Alum (M4-6), described in figure 6. Plasma were obtained at week 21 after the beginning of vaccination. The ELISA method and the cut-off determination are described in figure 4. Results reported in the histogram (A) refer to samples diluted 1:50 and to each peptide and are the average of the optical densities at 405 nm of the readings from duplicate wells. Results in the histogram (B) refer to the titration of plasma shown in (A) and are expressed in ordinate as the highest reciprocal dilution of plasma which at 405 nm gave a reading higher than the cut off value.

FIG. 9. Analysis of the presence of a anti-Tat specific CTL response. The assay was carried out as described in Table 5. In the figure it is shown an example at the 36th week for monkey M1, injected subcute with 10 μg of Tat and RIBI as described in figure 6. Squares (control) correspond to the cells incubated with unpulsed Bicl target cells with Tat; rhombs correspond to the cells incubated with the Bicl target cells pulsed with Tat (1mg/250.000 cells).".

Note 35: page 22 of the description: line 19: change "7" into "10".

Note 36: page 22 of the description: line 21: after "BSA" erase "and" and insert ".

Note 37: page 23 of the description: line 2: after "M2" add ", inoculated with 10 μg of Tat protein and RIBI and described in Figure 6,".

Note 38: page 23 of the description: after line 4 go on a new line and insert "**FIG. 11.** Humoral anti-Tat IgG response specific response in one monkey (M1) inoculated intradermally with 200 μg of DNA, the pCV-Tat plasmid resuspended in 150 μl of PBS-A, in two sites close to the axillary lymph-nodes; one monkey (M2) was injected with 500 mg of pCV-Tat, resuspended in 250 μl of PBS-A, intramuscular in two sites of the back; the control monkey (M3) was not inoculated but received, as a control of specificity, repeated skin tests with Tat. Monkeys were injected with PCVTat at time 0 and after 5, 10, 15, 22, 27, 32 and 37 weeks. Finally, after 42 weeks, monkeys were boosted with recombinant Tat protein (15 μg) resuspended in 200 μl of Iscom and 300 μl of PBS. Antibodies were evaluated at weeks 2, 5, 10, 15, 22, 27, 32, 37, 42, 48 and 58. Anti-Tat antibody response in plasma (diluted 1:50) was analyzed by Elisa as described in figure 2. Results reported in the histogram are the average of the optical densities at 405 nm of the readings of duplicate wells. (A) shows the results obtained from the two monkeys vaccinated with 200 (M1) and 500 (M2) μg of pCV-Tat plasmid. (B) shows the results of the control monkey (M3).

FIG. 12. Titration of anti-Tat antibodies in plasma from monkey M2 inoculated intradermally with 200 mg of pCV-Tat plasmid. The ELISA is described in figure 2. Results in ordinate are expressed as the reciprocal of the highest dilution which at 405 nm gave a reading higher than the cut off value for each dilution.

FIG. 13. Analysis of the specific umoral anti-Tat IgG response in three monkeys (M9-11) inoculated intramuscularly with 1 mg of pCVTat DNA plasmid and in one control monkey (M12), inoculated with 1 mg of control vector pCV0. DNA was resuspended in 1 ml of PBS-A and injected intramuscularly in two sites of the back. Monkeys were inoculated at time 0 and after 6, 11, 15, 21, 28 and 32 weeks. At the 36th week monkeys M9-M11 received a boost with 15 μg of recombinant Tat protein resuspended in 200 μl of Iscom and 300 μl of PBS. The

presence of anti-Tat antibodies was evaluated at weeks 2, 6, 11, 15, 21, 28, 32, 36, 40 and 44. Elisa and cut-off determination are described in figure 2.".

Note 39: page 23 of the description: line 5: change "8" into "14".

Note 40: page 23 of the description: line 9: change "immuno-magnetic methods" into "paramagnetic beads".

Note 41: page 23 of the description: line 10: change the word "part" into "half".

Note 42: page 23 of the description: line 11: change the word "part" into "half".

Note 43: page 23 of the description: line 17: change "9" into "15".

Note 44: page 23 of the description: line 19: after the word "obtained" erase from "with the methods ..." to "in example 5." and insert " from 4 monkeys with the method described in Figure 14, were stimulated as reported in Example 7.".

Note 45: page 23 of the description: line 1: change "5" into "7" and insert "**FIG. 16.** Functional characterization of dendritic cells (DC) obtained from monkey's peripheral blood. A. ^{3}H-Thymidine incorporation at day 4 of allogeneic mixed leukocyte culture (AMLR) to compare the antigen-presenting-function (APC, determined as the induction of proliferation of allogeneic T cells) of DC and macrophages (Mø) obtained from PBMC of *Macaca fascicularis* peripheral blood after separation on Percoll gradient and adherence on plastic. Non-adherent cells were removed and adherent cells were induced to mature into DC by adding GM-CSF (200 ng/ml) and IL-4 (200 U/ml) every 3 days. Half of the culture medium (RPMI, 10% FCS) was removed and substituted with fresh medium every 3 days. After 6-7 days a morphological change of cytokine-induced cells was observed, which acquired a typical DC phenotype (loss of adherence, clustering, fingers), also verified by determining typical membrane markers (data not shown). Monocytes were not cytokine-induced and were cultured in the same medium, that was replaced every 3 days. The cells maintained the monocyte-macrophage characteristics, such as the adherence. At day 7 both cell populations were challenged with T-lymphocytes from a human blood donor, purified by Ficoll and Percoll gradient and by adherence and then frozen.

Human T-lymphocytes cell proliferation assays were carried out in a 48-well plate with DC or Mø of monkeys. Five hundred thousand T lymphocytes were

stimulated with 5000 DC or Mø (T:APC ratio = 100:1). The culture was maintained for 4 days and fixed aliquots of the cell suspension were transferred in 96-well plates, in triplicate. 1 μCi per well of ^{3}H-Thymidine was then added for 16 hours, and the counts per minute (cpm) of the incorporated precursor were determined with a scintillation counter.

B. Cells presenting Ag (APCs) such as DC and Mø, obtained as reported in figure 16A, were challenged with T lymphocytes from another monkey, obtained as reported above for the human donor, to verify the greater ability to present the Ag, which is a typical characteristic of the DC as compared to Mø. APCs were added at scalar concentrations to T lymphocytes in order to evaluate the proliferative responses obtained at different T:APCs (DC or Mø) ratios.".

Note 46: page 40 of the description: last line: after the word **"specific"** insert **"and protective efficacy in immunization against virus challenge".**

Note 47: page 41 of the description: line 2: after the word "cellular)" insert "and protecting against virus challenge"

Note 48: page 41 of the description: line 14: after the word "have" erase from "inoculated..." to "not inoculated..." and insert "evaluated the tolerability, the safety and the ability to elicit a specific immune response (humoral and cellular). Thus, 3 monkeys were inoculated according to the following schedule: monkey 1 (M1) was inoculated with the recombinant Tat protein (100 mg), resuspended in 250 ml of autologous serum and 250 ml of RIBI, by the subcute route in one site; monkey 2 (M2) was inoculated with the recombinant Tat protein (10 mg), resuspended in 250 ml of autologous serum and 250 ml of RIBI, by the subcute route in one site; and monkey 3 (M3) was the control monkey not inoculated.".

Note 49: page 41 of the description: last line: after the value "22" erase "and 27 weeks" and insert "27, 32 and 37 weeks. The immunization schedule was interrupted at week 37 for monkey M1 and at week 41 for monkey M2. Animals were sacrificed to study the immunological parameters in several organs and tissues (spleen and lymph nodes), such as the evaluation of the presence of a proliferative response to Tat, of CAF activity and of CTL presence. CAF activity is the antiviral activity mediated by CD8+ lymphocytes, neither MHC-restricted nor

cytolytic.".

Note 50: page 42 of the description: line 6: erase "IL-15".

Note 51: page 42 of the description: line 8: erase "and CD45RO".

Note 52: page 42 of the description: line 9: after the word "activity" erase from "CTL and NK..." to "(CAF) and insert "specific cytotoxic activity (CTL), the presence of antiviral activity (CAF), and the presence of total antiviral activity (TAA) mediated by PBMC and by autologous serum.".

Note 53: page 42 of the description: line 13: erase from "This experiment..." to "non inoculated" of page 45 of the description and insert "The results of this experiment are as follows. No alterations of the chemo-physic, haematologic and behavioristic parameters were observed. In vaccinated and control monkeys, signs of inflammation and neo-vascularization were not detected at the sites of inoculation. These results indicate that the Tat protein was well tolerated by the animals and that was non-toxic at the administered doses and at the given inoculation route. In monkeys M1 and M2 the presence of antibodies of the IgG type specific anti-Tat were detected at week 5 after the first inoculation. At week 37, anti-Tat IgG were detectable up to 1:6400 plasma dilution in both monkeys, and, at week 41, up to 1:12.800 plasma dilution in monkey M2. The results are shown in figures 2 and 3. In the control monkey M3, anti-Tat antibodies with low titers were detected, likely elicited by the repeated inoculations of low amount of Tat that was injected in this monkey to control the specificity of the skin test reactions. In monkeys M1 and M2, anti-Tat antibodies were mainly directed against the amino-terminal region (aa 1-20) of Tat, with a titer of 1:3200 (figure 4). In monkey M2, vaccinated with 10 μg of Tat, antibodies directed against aa 36-50 and 46-60 of Tat were also detected, with titers of 1:50 and 1:100, respectively (figure 4). The ability of monkeys' serum to neutralize Tat was determined by means of in vitro assays that measured the inhibition of the rescue of HIV-1 replication in HLM-1 cells after the addition of exogenous Tat protein, as previously described [Ensoli et al., J. Virol. 67:277 (1993)]. These assays demonstrated that plasma from monkeys M1 and M2 diluted 1:2 and obtained at week 27 after the first inoculation, blocked virus replication induced by exogenous

as determined by quantification of p24 antigen in the culture supernatants. Conversely, preimmune plasma from the same monkeys did not block Tat activity (Table 4).

TABLE 4

Neutralizing activity of monkeys' plasma on the rescue of virus replication induced by extracellular Tat [a]

Samples	Inhibition (%)
Tat (30 ng/ml) + Preimmune M1	0
Tat (30 ng/ml) + Preimmune M2	0
Tat (30 ng/ml) + Immune M1	79,12
Tat (30 ng/ml) + Immune M2	100

[a]The neutralizing activity of plasma was determined in HLM-1 cells (HeLa-CD4$^+$ cells containing an integrated copy of an HIV-1 provirus defective in the tat gene). HLM-1 cells were seeded at 6 x 10^5 cells/well in 24-well plates and incubated at 37°C for 16 hours. Cells were washed twice with PBS, containing 0.1 % of bovine serum albumine (BSA), and cultured for 48 hours with medium (0.3 ml) in the presence of recombinant Tat protein and an equal volume of the animal plasma, withdrawn at time 0 (preimmune plasma) or at week 27 (immune plasma). Negative controls were represented by cells treated only with the preimmune plasma pooled together, with the immune plasma pooled together or with PBS containing 0.1% BSA (PBS + 0.1%BSA), without Tat. In all control samples no effects were observed on the rescue of virus replication. Each plasma was tested in duplicate. The presence of virus released by the cells was assayed by quantitation of p24 Gag antigen, using a commercial p24 antigen capture ELISA kit (NEN-Dupont). The results are expressed as the percentage of inhibition of virus rescue [measured for each plasma as the average value of p24 (pg/ml) in two wells] by the immune plasma as compared to the preimmune plasma (0% inhibition). Monkeys M1 and M2 were vaccinated with the recombinant Tat protein (100 mg or 10 mg, respectively) resuspended in 250 ml of autologous serum and 250 ml of RIBI, and injected by the subcute route in one site of the back near the axillary limph nodes.

The results indicate the presence of a proliferative response to Tat at week 22 (Table 5) in monkeys vaccinated with the recombinant Tat protein, being higher in

monkey M2 that received 10 mg of recombinant Tat protein at each boost.

Table 5

Proliferative response to Tat[a]

Monkey	Stimulus	Weeks from the primary immunization				
		15	22	27	32	37
M1	PHA	15.3	13.9	19.9	40.6	3.2
	TT	1.2	4.7	2.1	3.8	2
	Tat	0.8	2.4	1.1	1.3	0.6
M2	PHA	8.1	11.6	17.1	16.8	1.7
	TT	2	3.8	1.7	1	0.6
	Tat	0.9	3	1.4	1.2	0.6
M3	PHA	5.1	19.9	18.2	6.6	8.1
	TT	7.2	6.2	5.5	2.8	5.6
	Tat	2.1	1.4	1.3	0.7	0.9

[a]*Lymphocytes from peripheral blood isolated by Ficoll density gradient were plated at a concentration of 2×10^5 cells/well in triplicates in 96-well plates, cultured in RPMI-1640 supplemented with 10% fetal calf serum and stimulated with Tat (1 or 5 mg/ml), PHA (4 mg/ml) or Tetanus Toxcid (TT) (10 mg/ml) to which all monkeys were vaccinated. Unstimulated controls were incubated in RPMI, 10% FCS medium. The increase of cellular proliferation was measured at day 5 by ^{3}[H]-thymidine incorporation as previously described [Ensoli et al., IV International Conference on AIDS, Stockholm, 1:241 (1988); Cafaro et al., AIDS Res. Hum. Retrov. 7:204 (1991)]. Results are expressed as stimulation index and were calculated as follows: average of the test sample (cpm)/average of control (cpm). Values greater than 2 were considered positive. Monkeys M1 and M2 were immunized subcute with 100 mg or 10 mg respectively of recombinant Tat resuspended in 250 ml of autologous serum and 250 ml of RIBI. M3 represents a control monkey.*

The results in Table 6 show the absence of cytotoxic T lymphocytes (CTL) in monkeys M1 and M2 immunized with recombinant Tat.

TABLE 6

Analysis of cytotoxic activity to Tat (CTL)[a]

Monkey	Week	Target : Effector ratio						CTL activity
		1:50	1:25	1:12.5	1:6.25	1:3.125	Media	
M1	41	0	0	0	0	0	0	-
M2	41*	0	0	0	0	0	0	-
M3	41	0	0	0	0	0	0	-

[a]*The effectors were prepared as follows. PBMCs isolated by Ficoll density centrifugation were resuspended at a concentration of 1×10^7 cells/ml in RPMI 1640 supplemented with 10% FCS and seeded in a 24-well plate (500 µl per well) for 12 hours at 37°C in the presence or not of Tat (1 µg/well). One day later, the cells incubated without Tat were centrifuged at 1500 rpm and resuspended in 50 µl of RPMI 1640 supplemented with 10% FCS, incubated for 3 hours at 37°C with 1 µg of Tat, washed, resuspended in 500 µl of fresh medium and added to the well containing the PBMCs stimulated with Tat the day before. After additional 24 hours the cells were diluted with 1 ml of medium containing IL-2 (2 U/ml) and cultured for 14 days at 37°C. Autologous B lymphocytes isolated from each monkey before the vaccine protocol were used as target cells (BlcI). To this aim, PBMCs isolated by Ficoll density centrifugation at day -35 were seeded at a concentration of 3×10^5 cells/well in a 96-well plate and cultured for 2 or 3 weeks in the presence of 50% of a medium collected from a cell line that produces Papiovirus as previously described [Chen et al., J. Immunol. 149:4060 (1992)]. Ten B cell lines obtained for each animal were expanded and frozen. To test the toxicity, the Delfia Cytotoxic Test (Wallac, Turku, Finland) based on the time resolved fluorescence was used [Blomberg et al., J. Immunol. Methods 160:27-34 (1993); Blomberg et al., J. Immunol. Methods 168:267-273 (1994); Blomberg et al., J. Immunol. Methods 193: 199-206 (1996)]. To this aim, BLCL were cultured at a concentration of 1×10^6 cells/200 µl of RPMI 1640 supplemented with 10% FCS containing 4 µg of Tat for 12 hours at 37°C. BlcI were washed and resuspended in 1 ml of medium supplemented with 10% FCS containing 5 µl of fluorescence enhancing ligand and incubated for 15 min at 37°C according to the*

manufacturer's instruction. After 5 washings, Blc' were resuspended at a concentration of $5x10^4$ cells/ml and promptly centrifuged at 1800 rpm in order to harvest the supernatant that was used to measure the background level. PBMCs (Effectors) were seeded in duplicate at a concentration of $2.5x10^4$ cells/100 µl in medium containing IL-2 and properly diluted in a 96-well plate. $5x10^3$ of target cells/100 µl (cultured with or without Tat) were added to each well. Target:Effector ratios were 1:50, 1:25, 1:12.5, 1:6.25, 1:3.125. PBMCs and target cells (Tat-pulsed or unpulsed) were incubated for 2 hours at 37°C with: 1) 20 µl of 5% Triton to measure the maximum release, 2) 100 µl of growth medium to detect the spontaneous release, 3) 200 µl of supernatant from target cells to detect the background level. At the end of the incubation period the plates were centrifuged, 20 µl of each supernatant were transferred into a new plate and incubated in the presence of 200 µl of an Europium solution included in the kit. The fluorescence was measured after 20 min incubation with a time resolved fluorescence reader (Victor, Wallac, Turku, Finland). Specific CTL activity was measured as following: % specific release= [(average of sample detection - background) - (spontaneous release - background)]/[(Maximum release - background) - (spontaneous release - background)] x 100. The test was considered positive when the Tat specific-release was higher than 4% at most of the Effector : Target ratios tested. 4% is an arbitrary value established on the basis of previous control experiments . ND, not determined. Monkey M2 was immunized subcute with 10 mg of recombinat Tat resuspended in 250 ml of autologous serum and 250 ml of RIBI. M3 represents a control monkey.

** Lymphocytes were isolated from peripheral lynph nodes when M2 had been sacrificed.*

Moreover, the results demonstrate, at weeks 22, 27 and 37, the presence of soluble antiviral activity mediated by CD8+ T lymphocytes (CAF), measured as the ability of cell supernatants from monkeys CD8+ T lymphocytes to inhibit acute infection of the chimeric virus SHIV 89.6P in CEMx174 cells, or to control reactivation of HIV-1 chronic infection in OM-10-1 cells (Table 7). CAF activity was generally observed in vaccinated monkeys as compared to control animals.

TABLE 7

Analysis of the presence of soluble antiviral activity mediated by CD8+ T lymphocytes (CAF)[a]

Monkey ID	Week after the primary immunization	% inhibition of viral replication	
		Acute infection	Chronic infection
M1	22	89,5	ND
	27	62	61,7
	37	ND	ND
M2	22	44	ND
	27	54	27
	37	48	53
M3	22	24	ND
	27	37	22
	37	75	23

[a] *Peripheral blood mononucleated cells (PBMC) from monkeys vaccinated with 100 μg (M1) and 10 μg (M2) of recombinant Tat protein and from a control monkey (M3), that was not vaccinated, were isolated by Ficoll density gradient. CD8+ T lymphocytes enriched cultures were isolated from PBMC by anti-CD8 magnetic beads (Dynabeads, Dynal, Norway) according to manufacturer's instructions. The purity of the cultures was controlled by FACS analysis using a series of antibodies directed against specific cellular markers (CD3, CD4, CD8). CD8+ enriched cultures were seeded (in duplicate) at 5 x 10^5 cells/500 μl per well in 48-well plates, previously coated with an anti-CD3 monoclonal antibody (2.5 μg/ml, BioSource International, Camarillo, CA) for 12 hours at 4°C, and grown in RPMI 1640, containing 10% fetal bovine serum and IL-2 (20 U/ml). 250 μl of medium were collected every three days, for two weeks, and substituted with an equal volume of fresh medium. Cell supernatants were centrifuged, filtered (0.45 μm) and stored at –80°C. Cell supernatants derived from all time points, with the exception of the first one, were pooled and the presence of antiviral activity was tested as their ability to inhibit viral replication in two systems, represented by acute and chronic infection, respectively. For the acute infection system, the CEM x 174 cell line was used, which derives from the human B cell line 721.174 fused with the human T cell line CEM [Salter et al., Immunogenetics 21:235 (1985)].*

Cells (2 x 10^5) were incubated in polypropilene tubes with or without 200 µl of the supernatants, prepared as described above, for 2 hours at 37°C and then infected with 500$TCID_{50}$ of the chimeric virus SHIV 89.6P for 3 hours at 37°C. Cells were washed 3 times with fresh medium, seeded at 2 x 10^4 cells per well, in 96-well plates, and incubated in 200 µl with (treated cells) or without (untreated cells) different volumes (50 µl, 5 µl and 0.5 µl) of culture supernatants derived from CD8+ T lymphocytes of monkeys injected with the vaccine or the control monkey. After infection, aliquots of culture supernatants were collected every three days and substituted with an equal volume of complete medium previously added with the CD8+ culture supernatant from vaccinated and control monkeys. The results shown in the Table correspond to day 7 after infection and are expressed as percentage (%) of inhibition of viral replication of cells treated with CD8+ culture supernatants derived from vaccinated monkeys as compared to untreated cells. Viral replication was determined by measuring the RT values, as described [Goletti et al., J. Virol. 69:2540 (1995)], or the p27 Gag values by ELISA, in the cell supernatants collected at each time point. For the chronic infection system OM-10-1 cell line was used [Butera et al., J. Virol. 65:4645 (1991); Butera et al., J. Virol. 68:2726 (1994)], which represents a human T lymphocytic line chronically infected by HIV-1. Cells were seeded (in duplicate) at 5 x 10^4 cells/200 µl per well, in 96-well plates, in the presence of anti-TNFβ antibodies (40 µg/ml), with or without different volumes (50 µl, 5 µl, 0.5 µl) of cell supernatant from CD8+ T lymphocytes derived from vaccinated or control monkeys. Cells were activated to proliferate by PMA (10^{-7} M). After 24 hours, aliquots of culture medium were collected to determine viral replication by measuring RT or p24 Gag levels by ELISA. The results are represented as % of inhibition of reactivation of infection in treated cells as compared to untreated cells. The results of acute and chronic infection shown in the Table refer to cells treated with 5 µl of supernatant derived from CD8+ cell cultures. ND: not done.

Analysis of the delayed hypersensitivity (DTH, response of Th1 type) by means of a skin test showed that both the vaccinated (M1 and M2) and control (M3) monkeys were negative (Table 8).

TABLE 8

Skin-test to Tat[a]

Weeks after the primary immunization	Monkeys		
	M1	M2	M3
10	-	-	-
15	-	-	-
22	-	-	-
27	-	-	-
32	-	-	-
37	-	-	-

[a]*Tat (1 and 5 mg) in 150 ml of PBS-0.1% BSA or the buffer alone were inoculated by the intradermal route in a dorsal area previously shaved of the vaccinated and control (control of specificity of the response) monkeys, at weeks 10, 15, 22, 27, 32 and 37 following the first immunization. Monkeys M1 and M2 were vaccinated with recombinant Tat protein (100 mg and 10 mg, respecitvely) in 250 ml of autologous serum and 250 ml of RIBI, injected by the subcute route in one site. Monkey M3 is a control monkey that has not been vaccinated. The appearance of a nodular erythema after 48-72 hours was suggestive of a delayed hypersensitivity reaction (DTH): ++, Ø ≥ 5 mm; +, Ø ≥ 1-4 mm; +/-, erythema without hardening; -, Ø < 1 mm.*

Note 54: page 45 of the description: line 14: after the word "mediated" insert "with antiviral effects.".

Note 55: page 45 of the description: line 15: change "2" into "M2".

Note 56: page 45 of the description: line 18: erase from "In an experiment..." to "induced by" and insert "Based on these results, a second pilot experiment was designed in order to determine the effects of immunization with 10 µg of".

Note 57: page 45 of the description: line 21: change "1-3" into "M1-3".

Note 58: page 45 of the description: line 23: change "4-6" into "M4-6".

Note 59: page 45 of the description: last line: change "7" into "M7".

Note 60: page 46 of the description: line 1: change "8" into "M8" and after the word "autologous" add: "(control)".

Note 61: page 46 of the description: line 5: after "11" erase from "and 15 weeks..." to "pilot experiment." and insert "15, 21, 28 and 32 weeks. At week 36 monkeys

M1-6 received the last bbost with recombinant Tat protein (15 µg) in 200 µl of Iscom (immune stimulating complex) and 300 µl of PBS. ISCOM is an adjuvant consisting of quil A saponin, cholesterol and phospholipids which increase humoral and cell-mediated immune response [Morein et al., AIDS Res. Hum. Retrov. S10:S109 (1994); Lövgren et al., Vaccine 14:753 (1996)]. Monkeys M7 and M8 were injected at the same time points only with adjuvants. At each vaccination point and at weeks 40, 44 and 50, ml of blood were withdrawn from the animals to analyze the clinical and immunological parameters described in the previous pilot experiment. Moreover, urine samples and vacinal swabs were collected to analyze the presence of Tat specific secretory IgA. In order to evaluate the protective effect of Tat immunization against the infection, vaccinated and control monkeys, at week 50, were challenged, by intravenously inoculating 10 MID_{50} (50% monkey infectious dose), with the chinmeric "simian/human immunodeficiency virus" (SHIV), strain 89.6P, containing the HIV-1 tat gene, previously grown and titered in *Macaca fascicularis* [Reimann et al., J. Virol. 70:3189 (1996); Reimann et al., J. Virol. 70:6922 (1996); Karlosson et al., J. Virol. 71:4218 (1997)]. After challenge, animals were monitored (every two weeks for the first month, every four weeks for the next three months and every 8 weeks up to 6-12 months) for virological parameters, such as plasma p27 antigemia and plasma and cellular viral load. To confirm taht infection had occurred, anti-SIV antibodies were also searched by means of a commercial kit used for the detection of anti-HIV-2 antibodies which recognizes also anti-SIV antibodies (Elavia Ac-Ab-Ak II kit, Diagnostic Pasteur, Paris, France).".

Note 62: page 46 of the description: line 19: after the word "available" erase "and relating to week 15 from the beginning of the vaccination,".

Note 63: page 47 of the description: line 2: after "from" erase "1:12.800 to levels higher than 1:50.000 (figures 4-6). Moreover" and insert "1:6400 to 1:25600 (figures 5-7). The antibodies essentially reacted with the amino-terminal region (aa 1-20) of Tat, with titers ranging from 1:1600 to 1:3200 (figure 8) as shown at week 22. Moreover, antibodies directed against aa 46-60 of Tat, with titers ranging from 1:100 to 1:200, were also detected (figure 8).

Note 64: page 47 of the description: line 5: after the words "treated with" erase from "the exogenous Tat protein..." to page 55, line 7 of the description and insert different amounts of exogenous Tat protein, as previously described in the first pilot experiment. Such tests demonstrated that plasma (diluted 1:2) from monkeys M1-6 at week 15 blocked viral replication induced by 30 ng/ml of exogenous Tat, as determined by the measurement of p24 antigen released into the culture medium. Conversely, the preimmune plasma of same monkeys ar time 0 or plasma from control monkeys did not block Tat activity (Table 9). Moreover, immune plasma (diluted 1:2) of monkeys M1-6, withdrawn at week 21, blocked virus replication induced by 60 ng/ml, 120 ng/ml, 240 ng/ml and 500 ng/ml of exogenous Tat. In particular, these plasma determined a 10-fold reduction of virus replication induced by very high doses of extracellular Tat (240 ng/ml and 500 ng/ml) (Table 9).

TABLE 9

Neutralizing activity of immune plasma on the rescue of virus replication induced by extracellular Tat"

Samples	Inhibition (%)
Tat (30 ng/ml)+ Preimmune M1	0
Tat (30 ng/ml)+ Preimmune M2	0
Tat (30 ng/ml)+ Preimmune M3	0
Tat (30 ng/ml)+ Preimmune M4	0
Tat (30 ng/ml)+ Preimmune M5	0
Tat (30 ng/ml)+ Preimmune M6	0
Tat (30 ng/ml)+ Immune M1 (week 15)	89,8
Tat (30 ng/ml)+ Immune M2 (week 15)	78,7
Tat (30 ng/ml)+ Immune M3 (week 15)	100
Tat (30 ng/ml)+ Immune M4 (week 15)	100
Tat (30 ng/ml)+ Immune M5 (week 15)	70,8
Tat (30 ng/ml)+ Immune M6 (week 15)	94,2
Tat (60 ng/ml)+ Preimmune M1	0
Tat (60 ng/ml)+ Preimmune M2	0
Tat (60 ng/ml)+ Preimmune M3	0
Tat (60 ng/ml)+ Preimmune M4	0
Tat (60 ng/ml)+ Preimmune M5	0
Tat (60 ng/ml)+ Preimmune M6	0
Tat (60 ng/ml)+ Immune M1 (week 21)	96,3
Tat (60 ng/ml)+ Immune M2 (week 21)	100
Tat (60 ng/ml)+ Immune M3 (week 21)	100
Tat (60 ng/ml)+ Immune M4 (week 21)	98,7
Tat (60 ng/ml)+ Immune M5 (week 21)	99
Tat (60 ng/ml)+ Immune M6 (week 21)	98,8
Tat (120 ng/ml)+ Pool preimmune M1-6	0
Tat (120 ng/ml)+ Immune M1 (week 21)	59,2
Tat (120 ng/ml)+ Immune M2 (week 21)	90,4
Tat (120 ng/ml)+ Immune M3 (week 21)	96,8
Tat (120 ng/ml)+ Immune M4 (week 21)	98,3
Tat (120 ng/ml)+ Immune M5 (week 21)	100
Tat (120 ng/ml)+ Immune M6 (week 21)	97,8
Tat (240 ng/ml)+ Pool preimmune M1-6	0
Tat (240 ng/ml)+ Immune M1 (week 21)	26,1
Tat (240 ng/ml)+ Immune M2 (week 21)	49,4
Tat (240 ng/ml)+ Immune M3 (week 21)	70,3
Tat (240 ng/ml)+ Immune M4 (week 21)	91,2
Tat (240 ng/ml)+ Immune M5 (week 21)	94,5
Tat (240 ng/ml)+ Immune M6 (week 21)	86
Tat (500 ng/ml)+ Pool preimmune M1-6	0

Samples	Inhibition (%)
Tat (500 ng/ml)+ Immune M1 (week 21)	32,7
Tat (500 ng/ml)+ Immune M2 (week 21)	38,9
Tat (500 ng/ml)+ Immune M3 (week 21)	57,5
Tat (500 ng/ml)+ Immune M4 (week 21)	89,4
Tat (500 ng/ml)+ Immune M5 (week 21)	72
Tat (500 ng/ml)+ Immune M6 (week 21)	71,8

The ability of anti-Tat plasma to neutralize Tat activity was determined in HLM-1 cells, as described in Table 4. Recombinant Tat protein (30 ng/ml, 60 ng/ml, 120 ng/ml, 240 ng/ml and 500 ng/ml) was added alone or together with an equal volume of monkey preimmune plasma taken at time 0 or at week 15 or 21 (immune plasma). Monkeys M1-3 were vaccinated with 10 mg of Tat in 250 ml of autologous serum and 250 ml of RIBI; monkeys M4-6 were vaccinated with 10 mg of Tat in 250 ml of autologous serum and 250 ml of Alum; two control monkeys were injected with RIBI (250 ml and 250 ml of autologous serum) (M7) or with Alum (250 ml and 250 ml of autologous serum) (M8). The results are represented as described in Table 4.

The ability of monkey plasma to neutralize the activity of extracellular Tat released by the cells during acute infection was tested in CEM x 174 cells infected with the chimeric virus SHIV 89.6P. At day 7 after infection virus replication was observed in 50% of control cells infected with SHIV and cultivated with the preimmune plasma of monkeys M1-6. Conversely, virus replication was not detected in infected cells that were grown in the presence of the immune plasma withdrawn at week 44 (Table 10).

TABLE 10

Neutralizing activity of immune plasma on transmission of virus infection [a]

Sample	p27 SHIV (pg/ml)
SHIV + Preimmune M1	Neg
SHIV + Preimmune M2	Neg
SHIV + Preimmune M3	1,080
SHIV + Preimmune M4	0,602
SHIV + Preimmune M5	1,169
SHIV + Preimmune M6	Neg
SHIV + Immune M1	Neg
SHIV + Immune M2	Neg
SHIV + Immune M3	Neg
SHIV + Immune M4	Neg
SHIV + Immune M5	Neg
SHIV + Immune M6	Neg

[a] *CEM x 174 cells (3 x 10^4 cells/150 µl) in 96-well plates were infected with the chimeric SHIV 89.6P virus (5 x 10^{-5} $TCID_{50}$/cell) for 2 hours at 37°C in RPMI 1640 containing 10% FCS. Cells were washed twice with RPMI 1640 and resuspended in 150 µl of complete medium added with 5% of the monkey preimmune plasma taken at time 0 or immune plasma (week 44) from animals vaccinated with recombinant Tat (10 µg) and RIBI (M1-3) or Alum (M4-6). Animal plasma were previously heated at 56°C for 30 min. and analyzed by ELISA to control anti-Tat antibody titers. Each serum was tested in duplicate. At days 3, 5 and 7 after infection 120 µl of culture medium were collected and substituted with an equal volume of fresh medium containing 5% of preimmune or immune plasma from monkeys M1-6. The ability of the plasma to neutralize extracellular Tat, released during acute infection, and to control the transmission of infection in vitro was determined by detecting the viral Gag p27 antigen in the culture medium by ELISA (Coulter International, Miami, FL). The results, represented as p27 values (pg/ml), correspond to the mean value of two wells for each serum at day 7 after infection.*

Moreover, a proliferative response to Tat was observed since week 11 (Table 11).

TABLE 11

Proliferative response to Tat [a]

Monkey	Stimulus	Weeks from primary immunization									
		0	11	15	21	28	32	36	40	44	50
M1	PHA	16.96	10.50	15.27	33.8	7.2	51,5	64.3	36,05	24	65,7
	TT	11.69	1.96	3.01	1.2	1,2	1.3	0,93	1.4	10.05	7,2
	Tat	1.12	1.55	0.52	1.7	0,8	0.8	0.6	0,7	9.27	4,7
M2	PHA	31,27	25,75	21.28	87.1	25.7	56	38.2	40,3	29,03	26
	TT	1.12	1.8	0.57	1,7	1.15	1.6	4.95	1.2	1,51	2,9
	Tat	1.08	3.65	6.22	14.14	3.5	1.8	4,1	1,9	7,67	13.2
M3	PHA	22.42	7.89	16.88	36.3	148.5	42	78.9	27	53,71	ND
	TT	11.43	0.95	1.71	1.25	1.2	1.1	1	1	1,81	ND
	Tat	1.65	2.69	18.82	23.51	12.03	0.9	1.3	0.5	23.85	ND
M4	PHA	3.88	20.77	15.22	83.7	18.6	35	38.2	45,2	57.47	15,8
	TT	2.85	4.49	9.07	6.9	15.8	3.7	3.8	5	19.77	6,6
	Tat	1.29	3.01	3.24	7.9	10.1	2.6	1.5	3.9	33.61	4,7
M5	PHA	6.25	5.74	16.74	72.2	7.45	41	56.5	32.9	33.85	12
	TT	2.31	1.07	4.84	3.9	0.9	0.83	1.4	1.24	10.22	1,95
	Tat	1.80	0.66	1.76	3.6	2.22	0.8	1.14	1.3	1.33	1.4
M6	PHA	11.96	17.94	2.77	29.4	7.3	25	8.3	6.85	18.01	5.2
	TT	4.14	1.71	0.13	1.7	10.34	1.3	1.8	1.1	2.49	0,9
	Tat	1.37	1.06	0.11	2.95	9.3	1.13	1.3	1	5.8	0.3
M7	PHA	21.65	20.30	37.93	17.6	17.9	75	12.9	34.8	41,81	27,5
	TT	0.97	0.80	0.88	1	0.6	1.04	0.6	0.4	1.11	1,1
	Tat	1.78	0.68	0.73	1	0.42	0.9	0.5	0.8	1.07	0,4
M8	PHA	26.51	67.09	16.38	14.9	17.2	28.2	18.95	20.6	28.61	13,6
	TT	1.20	10.78	0.20	1.6	0.62	0.8	1.2	0.9	1,11	2.1
	Tat	1.12	0.00	0.21	1.03	0.57	0.6	0.5	0.9	1.04	1

[a] Peripheral blood lymphocytes were isolated, activated with PHA (4mg/ml), then tetanus toxoid (TT) (10 mg/ml) and Tat (5 mg/ml) and assayed as described in Table 5. Monkeys M1-3 were inoculated with 10 mg of recombinant Tat protein in 250 ml of autologous serum and 250 ml of RIBI; monkeys M4-6 were inoculated with 10 mg of recombinant Tat in 250 ml of autologous serum and 250 ml of Alum; two control monkeys were inoculated with RIBI (250 ml and 250 ml of autologous serum) (M7) and with Alum (250 ml and 250 ml of autologous serum) (M8). ND, not done.

A strong cytotoxic T cell response (CTL) was detected in one monkey vaccinated with the Tat protein and RIBI (M1) and in two monkeys vaccinated with the Tat protein and Alum (M4 and M5), whereas a weaker CTL response was observed in monkey M6 immunized with Tat and Alum (figure 9 and Table 12).

TABLE 12

Analysis of CTL presence[a]

Monkey	Week	Target: Effector ratio						CTL activity
		1:50	1:25	1:12.5	1:6.25	1:3.125	Average	
M1	28	5.9	4.7	4.1	7.9	5.3	5.5	+
	36	ND	14.4	8.8	4.9	6.7	8.7	+
M2	28	ND	ND	ND	ND	ND	ND	ND
	36	ND	ND	ND	ND	ND	ND	ND
M3	28	0	0	0	0	0	0	-
	36	ND	0	0.6	0.5	2.0	0.7	-
M4	28	0	0	1.1	1.1	2.6	0.9	-
	36	ND	2.7	8.3	15	1.9	6.9	+
M5	28	4.9	3.9	4.7	5.5	1.7	4.1	+
	36	0	1	0	0	0	0.2	-
M6	28	0	2.6	1.1	7.2	7.2	3.6	+/-
	36	ND	0	0	0	0	0	-
M7	36	0	0	0	0	0	0	-
M8	36	0	0	0	0	0	0	-

[a] *The assay was performed as described in Table 6. Monkeys M1-3 were immunized with 10 mg of recombinant Tat in 250 ml of autologous serum and 250 ml of RIBI; monkeys M4-6 were inoculated with 10 mg of recombinant Tat in 250 ml of autologous serum and 250 ml of Alum; two control monkeys were inoculated with RIBI (250 ml and 250 ml of autologous serum) (M7) and Alum (250 ml and 250 ml of autologous serum) (M8). ND, not done.*

At week 44, the presence of total antiviral activity (TAA) was determined. TAA was measured as the ability of PBMC from monkeys vaccinated with Tat protein, cultured in the presence of autologous serum, to be resistant to SHIV 89.6P infection (Table 13).

TABLE 13

Analysis of the presence of total antiviral activity (TAA)[a]

Monkey ID	Days after infection	
	7	17
	Minimum infectious dose ($TCID_{50}$/cell)	Minimum infectious dose ($TCID_{50}$/cell)
M1	10^{-2}	10^{-2}
M2	10^{-4}	10^{-4}
M3	10^{-3}	10^{-3}
M4	10^{-2}	10^{-2}
M5	10^{-2}	10^{-2}
M6	10^{-3}	10^{-3}
M7	10^{-3}	10^{-3}
M8	10^{-4}	10^{-3}

[a] *PBMC were collected at week 44 from monkeys vaccinated with the recombinant Tat protein (10 μg) and RIBI (M1-3) or Alum (M4-6) and from control monkeys inoculated with RIBI (M7) or Alum (M8). PBMC, purified by Ficoll gradient and seeded in triplicate at 5 x 10^5/200 μl per well in 48-well plates, were grown in RPMI 1640 containing 10% FCS and 5% of autologous plasma in the presence of an anti-CD3 monoclonal antibody (5ng/ml) and IL-2 (2 U/ml), for 48-72 hours at 37°C. Cells were infected with serial dilutions of the chimeric virus SHIV 89.6P (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} TCID50/cell) for 2 hours at 37°C, washed 3 times with PBS-A and resuspended in 50% of conditioned medium and 50% of fresh medium at 5 x 10^5 cells/ml/well. At days 3, 7, 10, 14 and 17 after infection, aliquots of culture medium were collected and substituted with equal volumes of fresh medium. Virus replication was determined in cell supernatants by p27 Gag ELISA (Coulter International, Miami, FL). The results are shown as the minimum infectious dose of SHIV (TCID50/cell) able to infect the cells.*

The results demonstrate the presence of soluble antiviral activity mediated by CD8+ T lymphocytes (CAF) (Table 14). An overall increase of CAF activity was observed in vaccinated monkeys as compared to control animals.

TABLE 14

Analysis of the presence of soluble antiviral activity mediated by CD8+ T cells (CAF)[a]

Monkey ID	Weeks after the primary immunization	% inhibition of viral replication	
		Acute infection	Chronic infection
M1	0	8	30
	32	53	53
M2	0	36	0
	32	60	27
M3	0	0	37
	32	55	29
M4	0	45	0
	32	85	66
M5	0	41	0
	32	ND	ND
M6	0	49	18
	32	34	41.4
M7	0	39	39
	32	71	44
M8	0	37	0
	32	76	26.8

Analysis of the presence of soluble antiviral activity mediated by CD8+ T cells (CAF) from monkeys vaccinated with recombinant Tat protein (10 μg) and RIBI (M1-3) or Alum (M4-6), and from control monkeys inoculated with RIBI (M7) or Alum (M8). Acute infection was tested on CEM x 174 cells infected with SHIV 89.6P. The assay was performed as described in Table 7 and the results refer to day 7 after infection. The presence of CAF on the chronic infection system was tested in the U1 cell line [Folks et al., Science 238:800 (1987)], which is a promonocytic human cell line chronically infected by HIV-1. U1 cells, seeded at 1 x 10^4 cells/200 μl per well in 96-well plates, were incubated with PMA (10^{-8} M) to induce reactivation of HIV-1 infection, with or without different volumes (50 μl, 5 μl, 0.5 μl) of culture medium for CD8+ T lymphocytes derived from vaccinated and control monkeys. Three days after induction, the presence of HIV-1 in the culture medium was determined by viral RT assay or p24 Gag. The results are shown as % of inhibition of HIV-1 replication in cells treated with CD8+ T cells supernatants compared to untreated cells. The results of inhibition of acute and chronic infection refer to cells treated with 5 μl of CD8+ lymphocytes supernatants.

The production of cytokines (γIFN, IL-4, TNFα) and of the RANTES chemokine from PBMC of monkeys vaccinated with Tat and RIBI (M1-3) or Tat and Alum (M4-6) and control monkeys M7 and M8 was also determined (Table 15).

TABLE T5

Analysis of cytokine and chemokine production[a]

Monkey	Control				PHA				TT			
	γIFN	IL-4	TNFα	RANTES	γIFN	IL-4	TNFα	RANTES	γIFN	IL-4	TNFα	RANTES
M1	-/-	-/-	-/-	-/-	988/1096	-/-	948/-	1788/2564	-/-	-/3.8	-/-	Nd/Nd
M2	-/-	-/-	126/-	-/-	325/280	-/-	244/172	292/284	86/66	-/-	-/-	Nd/Nd
M3	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd
M4	-/-	-/-	-/-	-/-	426/66	-/-	98/-	-/284	-/-	-/-	-/224	Nd/Nd
M5	-/-	-/-	48/-	-/-	279/303	-/-	416/-	536/608	-/-	-/-	-/-	Nd/Nd
M6	-/-	-/-	-/-	246/-	255/137	-/-	-/-	1124/268	-/-	-/-	-/266	Nd/Nd
M7	-/-	-/-	-/-	-/-	150/169	-/-	40/-	1228/976	-/-	-/4	-/nd	Nd/Nd
M8	-/-	-/-	-/-	-/-	0/0	20/32	60/-	2160/1588	-/-	-/-	-/nd	Nd/Nd

Monkey	Tat (1 μg)				Tat (5 μg)			
	γIFN	IL-4	TNFα	RANTES	γIFN	IL-4	TNFα	RANTES
M1	-/-	-/-	-/-	-/-	-/-	-/-	16/-	-/-
M2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
M3	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd	Nd/Nd
M4	-/-	-/-	378/430	-/-	-/-	-/-	344/352	-/-
M5	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
M6	-/-	-/5	-/-	-/-	-/78	-/-	150/-	-/-
M7	-/40	-/3.3	84/-	-/-	-/-	-/3.2	-/-	-/-
M8	-/-	-/4.8	-/-	-/-	-/-	-/10	-/-	726/528

[a] *Analysis of the production of cytokines and chemokines after 48 and 96 hours culture (48/96) from PBMC of monkeys vaccinated with 10 mg of recombinant Tat and RIBI (M1-3) or Alum (M4-6). Control monkeys (M7 and M8) were inoculated with RIBI or Alum adjuvants, respectively. PBMC, withdrawn at week 44, were purified by Ficoll gradient, were seeded at 1 x 10^6 cells/ml per well in 24-well plates and grown in RPMI 1640 containing 10% FCS. PBMC were unstimulated (control), to evaluate the spontaneous release of cytokines and chemokines, or stimulated with PHA (2 µg/ml), the tetanus toxoid (TT, 5 µg/ml) or Tat (1 or 5 µg/ml). Aliquots of culture supernatants were collected 48 and 96 hours following stimulation to determine the presence of cytokines and chemokines, by means of commercial ELISA kits from BioSource International (Camarillo, CA, USA) to assay cytokines production, and from R&D Systems (Abdigdon, Oxon, UK) to evaluate RANTES production. The results are shown as pg/ml at 48 and 96 hours of culture (48/96), respectively. Cut-off values were (pg/ml): γIFN, 31.2; IL-4, 3.12; TNFα, 15.6; RANTES, 6.25. (-), values were lower than corresponding cut-off values. Nd: not done.*

Moreover, at week 15 five monkeys vaccinated with the recombinant protein (M2-6), showed a positive reaction to the skin test to Tat, with a strong delayed hypersensitivity reaction (Table 16 and figure 10). In monkeys 4 and 5 the skin test reaction was even stronger in the following weeks (Table 16).

TABLE 16

Skin-test to Tat[a]

Monkey	Weeks from the primary immunization						
	11	15	21	28	32	36	44
M1	-	-	-	-	-	-	-
M2	-	+	+	+/-	+/-	+	+/-
M3	+/-	+	+/-	+/-	-	-	-
M4	-	+	++	++	++	++	++
M5	+/-	+	++	+	++	++	+
M6	-	+	+/-	+/-	-	-	-
M7	ND	ND	ND	ND	ND	ND	ND
M8	ND	ND	ND	ND	ND	ND	ND

[a]*Tat (1 and 5 mg) in 150 ml of PBS-0.1% BSA or the buffer alone were inoculated by the intradermal route in a shaved area of the back of vaccinated monkeys. Control animals were not inoculated (ND, not done) at weeks 11, 15, 21, 28, 32, 36 and 44 after the first immunization. Monkeys M1-3 were vaccinated with 10 mg of recombinant Tat protein in 250 ml of autologous and 250 ml of RIBI; monkeys M4-6 were vaccinated with 10 mg of recombinant Tat protein in 250 ml of autologous serum and 250 ml of Alum; two control monkeys were inoculated with RIBI (250 ml and 250 ml of autologous serum) (M7) or Alum (250 ml and 250 ml of autologous serum) (M8). The presence of an erythematous nodule after 48-72 hours was suggestive of a delayed hypersensitivity reaction (DTH): ++, Ø ≥ 5 mm; +, Ø ≥ 1-4 mm; +/+, erythema without hardening; -, Ø < 1 mm.*

The post-challenge results indicate that 4/6 (67%) vaccinated monkeys were protected against infection with 10 MID_{50} of SHIV 89.6P, as shown by the results of the virological assays (Table 17). Particularly, p27 Gag antigen was not detected in plasma of monkeys M1, M2, M4 and M6, proviral DNA was not found by PCR in cells from these monkeys and cytoviremia was negative. Monkeys M3 and M5 were infected as shown by the presence of p27 in the plasma, by detection of proviral DNA in the cells and by a positive cytoviremia (Table 17). Both controls (M7 and M8) resulted infected. To further control the infectivity of the viral dose used for the challenge, another naive monkey (M13) was added to the control animals and infected with 2.85 MID_{50} of SHIV 89.6P (corresponding to a dose of virus 3.5-fold lower than the dose used for the challenge of the animals in the protocol). Monkey M13 resulted to be infected based on all the virological

assays. To confirm that the animals were exposed to the virus, the presence of antibodies against SIV antigens, encoded by the chimeric SHIV 89.6P virus (Gag, Pol, RT, Nef), was analyzed as already described in this Example. The presence of antibodies in the monkeys that were negative for the virological parameters confirm that these animals were exposed to the virus and indicates that an abortive infection of SHIV had occurred in these monkeys. Monkeys that showed low anti-SIV antibody titers will be studied for <u>in vitro</u> production of specific antiviral IgG (IVAP) [Zamarchi et al., AIDS Res. Human Retrov. 9:1139 (1993); Fiore et al., AIDS 5:1034 (19912)] according to the following method. PBMC (2x 10^6/well) are seeded in 24-well plates and stimulated with PWM (2 mg/ml, Sigma, St. Louis, USA). Following 7 days incubation (at 37°C in the presence of 5% CO_2 and 95% humidity) culture supernatants are collected to assay for anti-SIV antibody production as already described in this example. If necessary, lymphocytary proliferation tests in response to SIV antigens, in the same manner as HIV-1 Tat, as already described in this example.

TABLE 17

Analysis of virological parameters

Monkey	Days after challenge with SHIV89.6P							
	15				30			
	p27[a] (pg/ml)	DNA PCR (copies/ µg)[b]	Cytoviremia[c]	anti-SIV IgG[d]	p27 (pg/ml)	DNA PCR (copies/ µg)	Cytoviremia	anti-SIV IgG
M1	<20	<1	Neg	1:2	<20	<1	Neg	1:2
M2	<20	<1	Neg	1:2	<20	<1	Neg	1:2
M3	73.3	855	707.3	Neg	26.22	959	74.95	Neg
M4	<20	<1	Neg	1:2	<20	<1	Neg	1:2
M5	964	1147	>2818.3	Neg	20.8	>10[e]	78	1:2
M6	<20	<1	Neg	1:8	<20	<1	Neg	1:8
M7	287.8	838	707.9	>1:50	65.2	858	354.8	>1:50
M8	106.7	376	707.9	1:2	<20	311	44.6	1:5
M13	1876	+[f]	+[e]	ND	<20	+[f]	ND	1:1600

Analysis of the virological parameters after challenge of monkeys vaccinated with 10 mg of recombinant Tat protein and RIBI (M1-3) or Alum (M4-6). Control monkeys (M7 and M8) were inoculated with RIBI or Alum, respectively. Monkey M13 was a naive animal infected with 2.85 MID_{50} of SHIV 89.6P.

[a]The plasma antigenemia was evaluated by p27 Gag ELISA (Innogenetics, Belgium) and it is expressed as p27 values (pg/ml). Neg, the value was lower than the corresponding cut-off value (18 pg/ml).

[b]DNA was purified by whole blood using the QIAamp blood kit (Angiogen Gmbh and Qiagen Inc., Hilden, Germany). The quality of the DNA was controlled by PCR amplification of the β-globin gene, as described [Saiki et al., Science 230:1350 (1985)]. The presence of proviral DNA was analyzed by semiquantitative PCR amplification performed on 1 μg of cellular DNA using primers SG1096Ngag (corresponding at nucleotides 1096-1119 on SIVmac251 genome: 5'TTAGGCTACGACCCGGCGGAAAGA3') and SG1592CgagD (nucleotides 1569-1592 of SIVmac251 genome: 5'ATAGGGGGTGCAGCCTTCTGACAG3') which amplify a 496 base pair fragment of the SHIV gag gene, as described [Titti et al., Cell. Pharmacol. AIDS 3:123 (1996)]. To quantify the number of copies of proviral DNA, in each experiment a standard curve was prepared using the plasmid pCMRII-Δgag (containing a 100 base pair deletion in the gag gene of SIVmac251) as a template DNA and the primers described above that amplify a 396 base pair DNA fragment. Amplified DNAs were analyzed by electophoresis and quantified by densitometric analysis (Ultrascan LX Enhancer Laser, LKB, Bromma, Sweden). The relationship between the OD values and the number of molecules of the Δgag plasmid was correlated by means of linear regression analysis (Statgraphics, Manugistics, Inc. Cambridge, MA). The OD values were linear up to 1000 molecules (coefficient of correlation = 0.954± 0.026). The number of copies of SHIV proviral DNA/μg of cellular DNA was determined interpolating the OD values of each sample to the standard curve. The sensitivity of the assay was 1 copy of provirus/μg of DNA.

[c]Cytoviremia was determined in co-coltivation assays. To this aim 1 x 10^4 CEM/x 174 cells were cultivated in the presence of serial dilutions of PBMC under study

(a total of 12 dilutions, from 1 x 10^6 to 3,9 x 10^3 cells per well) in 96-well plates. At days 3, 7 and 10 after infection, 150 mL of complete medium were added to each well after having collected an equal volume of supernatants that were tested to assay the presence of p27 Gag by ELISA (Innogenetics, Belgium). The results were analyzed by means of the Reed and Muench formula to determine the number of productively infected PBMC per milion of total cells.

[d]The presence of antibodies against SHIV was determined on serial dilutions of animal plasma tested in duplicate using the Elavia Ac-Ab-Ak II kit (Diagnostic Pasteur, Paris, France), according to the manufacturer's instructions. The highest dilution at which plasma values were higher than the cut-off value is shown.

[e]Virus isolation was performed, instead of cytoviremia, for monkey M13. To this aim PBMC (3 x 10^6) from monkeys infected with different doses of SHIV 89.6P, purified by Ficoll, were cultivated with CEM x 174 cells (1 x 10^6) in 1 ml of medium containing 10% FCS. After 24 hours, cells were diluted at 1 x 10^6/ml and cultivated for three days. Two ml of medium were then collected and cells were re-seeded at 3 x 10^5/ml in 7 ml. The excess of cells was discarded. This procedure was repeated twice a week for 4 weeks. The presence of virus was determined by p27 Gag ELISA (Innogenetics, Belgium) and then by reverse trancriptase (RT) assay. Virus isolation was considered positive (+) when both assays (p27 and RT) were positive in 3 sequential samples.

Conversely, virus isolation was considered negative (-).

[f]A qualitative DNA-PCR was performed.

The virological data overlap the absolute number of CD4 lymphocytes that resulted dramatically reduced in infected monkeys (M3, M5, M7, M8) as shown in Table 18.

TABLE 18

FACS analysis of CD4+ and CD8+lymphocytes

Monkey	Days post challenge with SHIV89.6P											
	0			15			30			60		
	% (cells/µl)			% (cells/µl)			% (cells/µl)			% (cells/µl)		
	CD4+	CD8+	CD4+/ CD8+	CD4+	CD8+	CD4+/ CD8+	CD4+	CD8+	CD4+/ CD8+	CD4+	CD8+	CD4+/ CD8+
M1	32.1 (1490)	53 (2460)	0,6	ND	ND	ND	30,8 (2420)	57,3 (4500)	0,54	306 (2460)	57,9 (4670)	0,53
M2	27,7 (1550)	45.3 (2530)	0,6	ND	ND	ND	35,5 (2120)	43,7 (2610)	0,81	29.6 (2000)	42,4 (2870)	0,7
M3	33 (1120)	39.3 (1340)	0.84	ND	ND	ND	3.1 (190)	75.6 (4660)	0.04	6,2 (240)	70 (2750)	0,09
M4	16,6 (670)	68.3 (2740)	0,24	ND	ND	ND	17.25 (2050)	68,7 (8180)	0,25	15,5 (1520)	75 (7350)	0,21
M5	36,3 (2770)	43,9 (3350)	0.83	ND	ND	ND	1.1 (90)	82,3 (1300)	0,01	4,1 (480)	75,5 (8730)	0,05
M6	35,3 (1210)	43.4 (1490)	0,81	ND	ND	ND	35.9 (1240)	45.5 (1570)	0,79	37,8 (3700)	43,7 (4280)	0,86
M7	36,1 (1610)	31.3 (1400)	1,15	ND	ND	ND	7,4 (480)	66,1 (4260)	0,11	13,7 (860)	56,7 (3550)	0,24
M8	25,7 (850)	51,3 (1710)	0,5	ND	ND	ND	3,3 (210)	76,2 (4840)	0,04	8,1 (590)	64,9 (4670)	0,13
M13	40,5 (2590)	39,7 (2544)	1,01	38,4 (434)	33,6 (380)	1,14	35,1 (1721)	32,2 (1479)	116	3,1 (111)	62,3 (2225)	0,05

FACS analysis of CD4+ and CD8+ lymphocytes from monkeys vaccinated with 10 µg of recombinant Tat protein and RIBI (M1-3) or Alum (M4-6). Control monkeys (M7 and M8) were inoculated only with RIBI or Alum adjuvants, respectively. Monkey M13 was a naive animal infected with 2.85 MID_{50} of SHIV 89.6P. Analysis was performed by fluorescence-activated-cell-sorter (FACS) as described [Rosenberg et al., Int. Immunol. 9 (5):703 (1997)], using labeled-monoclonal antibodies (anti-CD4-FITC, BioSource; anti-CD8-PerCp, Becton-Dickinson). ND, not done.

The results before the challenge indicate that Tat as the immunogen, as well as RIBI and Alum as the adjuvants (or Iscom that was used as adjuvant in the last boost), were well tolerated by the animals and were non-toxic, confirming the results of safety and tolerability of the immunization with Tat obtained in the first pilot experiment. Moreover, these data confirm the observations of the first pilot experiment, supporting additional evidence to the fact that the recombinant Tat protein elicits a strong humoral and cellular anti-Tat response with antiviral effects in vitro and in vivo. The post-challenge results (4/6 protected monkeys) confirm the expectation of the in vitro results and indicate that an anti-Tat vaccine induces protection against infection and therefore against the disease. The follow-up of the two vaccinated and infected monkeys will clarify the effects of the vaccination on disease progression.

Example 5.

Inoculation in *Macaca fascicularis* of an anti-Tat DNA vaccine: analysis of safety, tolerability, specific immune response and efficacy of protection against viral challenge.

Note 65: page 55 of the description: line 17: change "PBS" into"PBS-A" and erase "(without Ca^{++} and Mg^{++})".

Note 66: page 55 of the description: line 18: after "are" insert "afterwards"

Note 67: page 56 of the description: erase from line 3 of page 56 to line 21, comprised, of page 58 of the description and insert "The tolerability, the safety, the ability to elicit a specific immune response (both humoral and cellular) and the efficacy of protection against the virus challenge following immunization with

pCVTat plasmid DNA were evaluated in cynomolgus monkeys (*Macaca fascicularis*). In a first pilot experiment, three monkeys were immunized according to the following schedule: monkey M1 was inoculated with 200 μg of pCVTat plasmid DNA in 300 μl of PBS by the i.d. route in 2 sites of the back, near the axillary lymph nodes (150 μl/site); monkey M2 was inoculated with 500 μg of pCVTat plasmid DNA in 500 μl of PBS by the i.m. route in 2 sites of the back (250 μl/site). At days 1 or 5 before the i.m. inoculation, 250 μl of physiological solution, containing 0.5% bupivacaine and 0.1% methylparaben, were injected in the two sites, previously marked, where plasmid DNA had to be inoculated. This was performed in order to increase the uptake and expression of DNA in the muscle [Danko et al., Vaccine 12:1499 (1994); Fine et al., Ann. Plast. Surg. 20:6 (1988)]. Monkey M3 was not inoculated and was used as a control animal. Ten ml of blood were withdrawn from all monkeys 42 and 35 days preceding the first inoculation for analysis of basal parameters. Monkeys were inoculated at time 0 and after 5, 10, 15, 22, 27, 32 and 37 weeks. Finally, at week 42, animals received the last boost with recombinant Tat protein (15 mg) in 200 μl of Iscom and 300 μl of PBS. Animals were observed daily for clinical parameters as described in Example 4. Moreover, 10 ml of blood were withdrawn the same day of inoculation as described in Example 4. The protective effect of vaccination was determined after challenge of the monkeys with 10 MID_{50} of SHIV89.6P, that was injected by the intravenous route at week 65. The post-challenge follow up, still ongoing, was performed as described in Example 4.

The results of this experiment are as follows. In two vaccinated monkeys and in the control monkey no alterations of clinical, haematological and behavioristic parameters were observed. Inflammatory signs or neovascularization in the site of injection were not observed. These results indicate that the pCV-Tat DNA was well tolerated by the animals and was non-toxic at the doses and inoculation routes used in the experiment. Monkey M1, vaccinated with 200 μg of DNA by the i.d. route, developed anti-Tat specific IgG antibodies since week 32 (Figure 11). The antibody titers (from week 32 to week 58) ranged between 1:100 and 1:800 (Figure 12). At week 37, epitope mapping analysis (performed as described

legend to Figure 4) showed that these antibodies were directed against Tat peptides, mapping at aa 1-20, aa 46-60 and aa 65-80, with titers of 1:200, 1:100 and 1:50, respectively (data not shown). In monkey M2, vaccinated with 500 μg of DNA by the i.m. route, anti-Tat antibodies were barely detected (with a 1:50 titer, not shown) for the entire period of the study. The results are shown in Figure 11.

The ability of plasma from monkey M1, vaccinated with 200 μg of DNA by the i.d. route, to neutralize Tat activity was tested by assaying the inhibition of the rescue of viral replication in HLM-1 cells incubated with exogenous Tat protein, as described in Example 4. This assay showed that the plasma of monkey M1, diluted 1:2, and obtained at week 37, reduced viral replication induced by 30 ng/ml of exogenous Tat. Conversely, the plasma of the same monkey obtained at time 0 (preimmune) did not block Tat (Table 19).

TABLE 19

Neutralizing activity of plasma on rescue of viral infection induced by extracellular Tat[a]

Samples	Inhibition (%)
Tat + M1 preimmune	0
Tat + M1 immune	51

[a] *The ability of anti-Tat antibodies to neutralize Tat activity was determined in HLM1 cells by adding 30 ng/ml of recombinant Tat protein, previously incubated with an equal volume of plasma obtained at time 0 (preimmune) or at week 37 (immune) from monkey M1, vaccinated with 200 μg of pCVTat by the i.d. route. The assay was performed and the results expressed as described in Table 4.*

The results shown in Table 20 demonstrate the presence of a proliferative response to Tat at week 42 in monkey M1 immunized with 200 μg of DNA by the i.d., whereas in monkey M2 this type of cellular response was not detected.

TABLE 20

Proliferative response to Tat [a]

Monkey	Stimulus	Weeks after the primary immunization							
		15	22	27	32	37	42	48	
M1	PHA	32,9	45	89,3	40.5	3,1	13,3	ND	1
	TT	0,8	2,7	1,5	1,3	0,6	9	1,2	
	Tat	0,9	1,7	1,2	1,1	1,1	5,9	1	
M2	PHA	11,7	18,5	21,8	32,2	1,1	6,2	7	
	TT	0,9	1,8	0,8	1,1	1	1,5	1,1	1
	Tat	0,8	1,4	0,9	1,1	1,1	1,3	1,1	1
M3	PHA	5,1	19,9	18,2	6,6	8,1	77,8	ND	2,1
	TT	7,2	6,2	5,5	2,8	5,6	36,8	1	2,1
	Tat	2,1	1,4	2,2	0,7	1,5	2,8	0,8	0,9

[a] *Proliferative blood lymphocytes were isolated, stimulated with PHA (4 mg/ml), tetanus toxoid (TT) and Tat (1 or 5 mg/ml) and tested as described in Table 5. Monkeys were vaccinated with 200 mg (M1) of pCVTat plasmid DNA by the i.d. route or with 500 mg (M2) of pCVTat DNAby the i.m. route. Monkey (M3) was not vaccinated. ND: not done.*

The anti-Tat cytotoxic activity (CTL) was detected in monkey M1 at week 48 and in monkey M2 at week 42. (Table 21).

TABLE 21

Analysis of Tat-specific cytotoxic activity (CTL)[a]

Monkey	Week	Target : Effector ratio						CTL activity
		1:50	1:25	1:12,5	1:6,25	1:3,125	Media	
M1	42	27,4	27,8	17,1	9,8	3,9	17,2	+
	48	ND	ND	21,3	0	11,7	11	+
M2	42	1,2	5,9	2,4	1	0	2,1	-
	48	ND	ND	ND	57	25,1	41	+
M3	42	0	0	0	1,2	0	0,6	-
	48	ND	12.4	4.2	0	0	0	+

[a] *The assay was carried as described in Table 6. Monkeys were vaccinated with 200 mg (M1) of pCVTat plasmid DNA by the i.d. route or with 500 mg (M2) of pCVTat plasmid DNA by the i.m. route. Control monkey (M3) was not vaccinated. ND: not done.*

The results shown in Table 22 indicate at week 52 the presence of total antiviral activity (TAA) in both monkeys vaccinated with 200 and 500 μg of DNA.

TABLE 22

Analysis of total antiviral activity (TAA) [a]

Monkey	Days post infection	
	7	17
	Minimum infectious dose ($TCID_{50}$/cell)	Minimum infectious dose ($TCID_{50}$/cell)
M1	10^{-4}	10^{-4}
M2	10^{-4}	10^{-4}
M3	10^{-8}	10^{-8}

[a] *The assay was performed as described in Table 13. Monkeys were inoculated with 200 mg (M1) of pCVTat plasmid DNA by the i.d. route or with 500 mg of pCVTat plasmid DNA by the i.m. route. Control monkey (M3) was not inoculated. PBMC were collected at week 52 from the primary immunization and were infected with SHIV 89.6P (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} $TCID_{50}$) The results are represented as the minimum infectious dose of SHIV ($TCID_{50}$/cell) that was still able to infect the cells.*

The results shown in Table 23 indicate the presence of soluble antiviral activity (CAF) mediated by CD8+ T lymphocytes, at week 22 and 27, in both vaccinated monkeys. This activity was higher than in the control monkey.

TABLE 23

Analysis of the CD8+ cell mediated soluble antiviral activity (CAF)[a]

Monkey	Weeks from primary immunization	% inhibition of viral replication	
		Acute infection	Chronic infection
M1	22	62	27
	27	56	25
M2	22	74	ND
	27	28	ND
M3	22	24	ND
	27	37	22

[a] *Analysis of the presence of soluble antiviral activity produced by CD8+ T cells (CAF) derived from monkeys inoculated with 200 μg (M1) and 500 μg (M2) of pCVTat plasmid DNA and from the non inoculated control monkey M3. The antiviral activity was assayed on acute and chronic infection in CEM x 174 cells infected with SHIV 89.6P and in OM-10-1 cells chronically infected with HIV-1, as described in Table 7. The results are represented as the percentage (%) of inhibition of viral replication in cells treated with supernatants from CD8+ T lymphocytes compared to untreated cells. The results of the acute and chronic*

infected, suggesting that, in regard to the immunization with DNA, the i.m. route is more effective than the i.d. inoculation. The control monkey M3 also resulted resistent to the infection. However, as previously described, this monkey, like the controls of the other experimental protocols, received repeated skin tests via for Tat in order to control the test specificity (Table 24), and anti-Tat antibodies, although at low titers (1:100), were detected since week 32 from the beginning of the immunization (data not shown). Finally, the proliferative response to Tat in this monkey showed a weak and sporadic reactivity to the antigen (Table 20). Although preliminary, these data indicate that the repeated i.d. injection of 5 or 6 mg of Tat could have resulted in the immunization of the animal and in the protection from challenge. Thus, the monkey M3 will be considered vaccinated and studied as such.

TABLE 25

Analysis of the virological parameters

Monkey	Days post-challenge with SHIV89.6P											
	15				30				60			
	p27[a] (pg/ml)	DNA PCR (copies/ μg)[b]	Cytoviremia[c]	Anti-SIV IgG[d]	P27 (pg/ml)	DNA PCR (copies/ μg)[b]	Cytoviremia[c]	anti-SIV IgG	p27 (pg/ml)	DNA PCR (copies/ μg)[b]	Cytoviremia[c]	anti-SIV IgG
M1	1796	1278	>2818,3	1:10	68.6	1048	353.9	1:50	<20	8	21.3	1:800
M2	<20	<1	Neg	1:10	<20	<1	Neg	1:50	<20	<1	Neg	1:10
M3	<20	<1	Neg	>1:50	<20	<1	Neg	>1:50	<20	<1	Neg	1:100

The monkey M1 had been immunized i.d. with 200 mg of DNA of the plasmid of pCVTat, the monkey M2 with 500 mg of DNA of the plasmid of pCVTat, i.m.. The monkey M3 (control) was not vaccinated. The virological parameters were evaluated as described in the legend to Table 17.

The FACS evaluation of the percentage and of the absolute number of the CD4 and CD8 lymphocytes confirmed the virological data, with a clear reduction (of approximately 4 folds) of the CD4 lymphocytes in the infected monkey, already at the first post-challenge analysis (day 30) and confirmed later on (day 60) (Table 26).

TABLE 26

FACS analysis of CD4 and CD8 subsets.

Monkey	Days post-challenge with SHIV89.6P virus											
	0			15			30			60		
	% (cells/µl)			% (cells/µl)			% (cells/µl)			% (cells/µl)		
	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+
M1	27.5 (940)	40.7 (1390)	0.68	ND	ND	ND	8,1 (250)	56.5 (1780)	0,14	9.5 (360)	69,7 (2650)	0,14
M2	22.2 (490)	36.8 (810)	0,6	ND	ND	ND	16,4 (580)	42.4 (1500)	0.39	10,9 (940)	52,7 (4560)	0,21
M3	28,7 (1170)	41,1 (1680)	0.7	ND	ND	ND	19,5 (970)	48.7 (2430)	0.4	17,9 (900)	5,.2 2620)	0,34

The analysis was performed as indicated in the legend to Table 18. The monkey M1 had been immunized i.d. with 200 mg of pCVTat plasmid DNA, the monkey M2 with 500 mg of pCVTat-DNA, i.m.. The macaque M3 (control) was not vaccinated.

Based on these results a second experiment was designed in which the effects of the immunization with the pCVTat plasmid DNA were evaluated in 3 monkeys (M9-M11) as compared to the control monkey (M12) that received the pCV0 plasmid. All animals were inoculated i.m. in 2 sites on the back with a total of 1mg of pCVTat (M9-M11) or of pCV0 (M12). Either 1 or 5 days before the vaccination, 250 µl of saline solution containing 0.5% of bupivacaine and 0.1% of methylparaben were inoculated into the two marked sites in which successively the plasmid would have been injected. The monkeys were vaccinated at time 0 and at week 6, 11, 15, 21, 28, and 32. A final booster was performed at week 36 with the recombinant Tat protein (15 mg) resuspended in 200 µl of Iscom and 300 µl of PBS. The animals were controlled every day for clinical parameters as described in the Example 4. Moreover, 10 ml of blood were drawn 9 days before the primary immunization and at every immunization, as described in the Example 4. In order to evaluate the protective effects of the vaccination, the monkeys were challenged at week 50 from the beginning of the immunization by intravenous injection of 10 MID_{50} of SHIV89.6P. The post-challenge follow-up is still ongoing and is performed as described in the Example 4.

The results of this experiment are the following. No modifications in terms of behavior, clinical parameters, and blood chemistry were noted both in the vaccinated and in the control animals. No signs of inflammation or vascular neo-formations were detected at the injection sites. These results confirm that 1 mg of the plasmid pCVTat DNA, injected i.m., was well tolerated and non-toxic. Anti-Tat IgG antibodies were detected since week 15 (figure 13), with titers ranging from 1:50 to 1:100 (data not shown). Moreover, a proliferative response to Tat was detected as early as week 2 in one monkey (M11) (table 27).

TABLE 27

Proliferative response to Tat [a]

Monkey	Stimulus	Weeks from primary immunization										
		2	6	11	15	21	28	32	36	40	44	50
M9	PHA	8.9	9.2	17.1	58.2	18	47,1	43.4	3,1	72,6	64,6	7
	TT	2.9	1.7	0.9	1	1.8	0.7	1.1	0,8	1	7	2,7
	Tat	0,4	0,5	0,6	1,5	1.6	0,9	1	0.7	1,1	7	1,9
M10	PHA	8.5	18	19.8	ND	10,1	2.2	14.7	15,2	4,4	8,4	ND
	TT	2.4	0.3	0.8	ND	1,1	0.6	1	0.9	0,6	6,4	ND
	Tat	1	0.3	0.7	ND	1,1	0.5	1	0.9	0,7	4,2	ND
M11	PHA	25.7	43.3		12.1	27.8	3.4	21.3	14.1	15,9	25,8	ND
	TT	4.2	1.9	1.3	0.9	1.1	3.6	1.2	0.8	0.3	1,8	ND
	Tat	5.1	0.8	1.6	0.7	1,1	1.1	1.2	0.7	0.7	3	ND
M12	PHA	28.7	30.9	41	50.7	30.8	7.6	43	22,6	34,6	19,9	55.1
	TT	3.2	1.6	0.9	5.2	1.6	1.6	1,3	1,1	1	0,7	3,1
	Tat	3.2	1.4	0.8	1.3	1	1.6	1	0,8	1	1,6	1,3

[a] *Peripheral blood lymphocytes were isolated, stimulated with PHA (4mg/ml), or tetanus toxoid (TT, 10 mg/ml), or Tat (1 and 5 mg/ml) and assayed as described in table 5. The monkeys were injected i.m. with 1 mg of either pCVTat plasmid DNA (M9-M11) or pCV0 plasmid DNA (M12, control). ND, not determined.*

Anti-Tat CTLs were detected at week 32 post-immunization in monkye 11 (Table 28).

TABLE 28

Analysis of the anti-Tat cytotoxic activity (CTL)[a]

Monkey	Week	Target : Effector ratio						CTL Activity
		1:50	1:25	1:12.5	1:6.25	1:3.125	Media	
M9	32	0	0	0	0	0	0	-
	50	4.2	0	0	0	0.9	1	-
M10	32	0	0	9.9	2.7	0	2,5	-
	50	3.5	0	2.3	0	0	1.1	-
M11	32	0	10.5	8.9	3.5	0.9	4.7	+
	50	0	0	0	3.8	0.3	0.8	-
M12	32	0	0	0	0	0	0	-
	50	0	0	0	0	0	0	-

[a]The assay was performed as described in Table 6. The monkeys were injected i.m. with 1 mg of either pCVTat (M9-M11) or pCV0 (M12, control) plasmid DNA.

PBMCs obtained from the monkey M11 at week 44 resulted resistent to in vitro infection with serial dilutions of the chimeric SHIV-89.6P virus by an assay that detects the presence of total antiviral activity (TAA). In fact, TAA is evaluated as the capability of PBMCs from monkeys vaccinated with pCVTat plasmid DNA, grown in the presence of autologous serum, to resist to the infection with serial virus dilutions. (Table 29)

TABLE 29

Analysis of the total antiviral activity (TAA)[a]

Monkey	Days post infection	
	7	17
	Minimum infectious dose ($TCID_{50}$/cell)	Minimum infectious dose ($TCID_{50}$/cell)
M9	10^{-2}	$>10^{-2}$ **
M10	10^{-3}	10^{-2}
M11	$>10^{-2}$ *	$>10^{-2}$ *
M12	10^{-2}	$>10^{-2}$ **

*[a]The assay was performed as described in Table 13. The monkeys were injected i.m. with 1 mg of either pCVTat (M9-M11) or pCV0 (M12, control) plasmid DNA. PBMCs were withdrawn at week 44 from the first immunization and infected in vitro with 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} $TCID_{50}$ of the SHIV-89.6P. The results are expressed as the minimum infectious dose of the SHIV ($TCID_{50}$/cell) still able to infect the cells. *No culture resulted infected at the highest SHIV concentration used in the assay (10^{-2} $TCID_{50}$/cell). **The cultures became negative on day 17 post-infection.*

The results shown in table 30 demonstrate the presence of the soluble antiviral activity (CAF) mediated by the CD8+ T lymphocytes in the vaccinated monkeys and in the control monkey (M12) injected with the empty vector (pCV-0).

TABLE 30

Analysis of the soluble antiviral activity (CAF) mediated by the CD8+ T lymphocytes (CAF) [a]

Monkey	Weeks from the primary immunization	% inhibition of viral replication	
		Acute infection	Chronic infection
M9	0	21	14,6
	36	77	2,6
M10	0	40	13,8
	36	67	25
M11	0	49	19
	36	42	14
M12	0	65	23
	36	62	14

[a]*Analysis of the presence of the soluble antiviral activity mediated by the CD8+ T lymphocytes (CAF). PBMCs were obtained from the three monkeys (M9-M11) injected with 1 mg of pCV-Tat plasmid DNA and from the control monkey (M12) inoculated with 1 mg of pCV0 plasmid DNA. The acute infection assay was carried out in CEMx174 cells infected with the SHIV-89.6P, as described in Table 7. The chronic infection assay was carried out in U1 cells chronically infected with the HIV-1, as described in Table 14. The results are expressed as the percentage (%) of inhibition of viral replication in cells cultured in the presence or in the absence of 5 µl of supernatants from CD8+ T cells.*

The production of cytokines (γIFN, IL-4, TNFα) and of the chemokine Rantes was evaluated at week 44 in PBMCs from both the vaccinated with pCVTat plasmid DNA and control monkeys (Table 31).

TABLE 31

Analysis of the production of cytokines and of chemokines [a]

Monkey	Control				PHA				TT			
	γIFN	IL-4	TNFα	Rantes	γIFN	IL-4	TNFα	Rantes	γIFN	IL-4	TNFα	Rantes
M9	-/-	-/3.5	-/-	-/-	312/204	-/-	250/-	536/2288	-/-	-/-	-/-	nd/nd
M10	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd
M11	-/-	-/-	-/-	-/-	420/183	-/-	388/-	4336/3124	-/-	-/-	-/nd	nd/nd
M12	-/-	-/3.2	-/-	-/-	430/932	-/-	-/-	1936/2576	-/-	-/-	218/nd	nd/nd

Monkey	Tat (1 μg)				Tat (5 μg)			
	γIFN	IL-4	TNFα	Rantes	γIFN	IL-4	TNFα	Rantes
M9	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
M10	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd
M11	-/-	-/4	-/-	544/368	-/-	-/3.5	-/-	2124/-
M12	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

The assay was performed as described in Table 15. The monkeys were injected im. with 1 mg of either Smide pCVTat (M9-M11) or pCV0 plasmid DNA (M12, control). PBMCs were withdrawn at week 44 after the first immunization. Results are shown as pg/ml of cytokines and Rantes detected at 48 and 96 hours (48/96) respectively. (-), the values were below the cut-off value. The cut-off values (pg/ml) were: γIFN: 31.2; IL-4: 3.12; TNF-α: 15.6; Rantes: 62.5. nd: not done.

The results show the presence of a weak reactivity to the skin tests in one monkey (M9) at week 11 (Table 32)

TABLE 32

Skin test to Tat [a]

Weeks from the primary immunization							
Monkey	11	15	21	28	32	36	44
M9	+/-	-	-	-	-	-	-
M10	-	-	-	-	-	-	-
M11	-	-	-	-	-	-	-
M12	ND	ND	ND	ND	ND	ND	ND

[a] *Tat (1 and 5 mg) in 150 ml of PBS, 0.1% BSA or the buffer alone were inoculated i.d. in a previously thrichotomized area of the upper back of the vaccinated animals but not in the control monkeys at weeks 11, 15, 21, 28, 32, 36, and 44 from the initial immunization (ND, not determined). The monkeys were injected i.m. with 1 mg of either pCVTat (M9-M11) or pCV0 (M12, control) plasmid DNA. The appearance, 48 to 72 hours later, of an erythematosus nodule indicated the presence of delayed-type hypersensitivity (DTH): ++, ø ≥ 5 mm; + ø 1-4 mm; ±, erythema without hardening; -, ø <1 mm.*

The post-challenge results indicate that all the vaccinated animals were protected from the infection with 10 MID_{50} of the SHIV89.6P, as indicated by the virological tests (plasma antigenemia, determination of the proviral DNA copy number, cytoviremia) that were all negative (Table 33). Moreover, the presence of anti-SIV antibodies in the monkey M11 indicated the exposure to the virus or an abortive infection. On the contrary, they were not detected in the remaining monkeys, therefore we decided to carry out the in vitro antibody production assay as well as the lymphoproliferative response to SIV antigens. In case these assays were negative, the monkeys will be inoculated with a higher dose of the virus, since even the control animal M12 resulted resistant to infection. This monkey had been vaccinated with the empty vector pCV0. Recent data from the literature have demonstrated the adjuvant role played by certain DNA sequences that are much more frequent in bacteria than in eukariotic cells, and that, similarly to lipopolysaccharide and mannose, represent a strong stimulus for the natural immunity [Roman et al., Nature Med. 3:849 (1997)]. Thus, it is conceivable that

the protection observed in the monkey M12 may be due to the induction of a non-specific antiviral immunity by these bacterial sequences, such as the production of IFNa, IFNb, IL-12, and IL-18, known to exert immuno modulant and antiviral functions. This is strongly suggested by the presence in this monkey of TAA (Table 29) and CAF (Table 30) antiviral activities in the absence of anti-Tat specific humoral and cellular immunity. In fact, these assays also measure non-antigen specific antiviral activities. This is confirmed by the fact that the naive monkey M13, inoculated with a 3.5 fold lower virus dose than that injected in the macaque M12 (Table 33), resulted infected. On the basis of this result the inventor plans to utilize the pCV0 vector or parts of it as an adjuvant.

TABLE 33

Analysis of the virological parameters

Monkey	Days post challenge with SHIV89.6P virus											
	15				30				60			
	p27[a] (pg/ml)	DNA PCR (copies/ μg)[b]	cytoviremia[c]	Anti-SIV IgG[d]	p27 (pg/ml)	DNA PCR (copies/ μg)	cytoviremia	Anti-SIV IgG	p27 (pg/ml)	DNA PCR (copies/ μg)	Cytoviremia	Anti-SIV IgG
M9	<20	<1	Neg	Neg	<20	<1	Neg	Neg	<20	<1	Neg	Neg
M10	<20	<1	Neg	Neg	<20	<1	Neg	Neg	<20	<1	Neg	Neg
M11	<20	<1	Neg	1:2	<20	<1	Neg	1:2	<20	<1	Neg	Neg
M12	<20	<1	Neg	Neg	<20	<1	Neg	Neg	<20	<1	Neg	Neg
M13	1876	+[f]	+[e]	ND	<20	+[f]	ND	1:1600	<20	43	ND	1:3200

The assays were performed as described in Table 17. The monkeys were injected i.m. with 1 mg of either pCVTat (M9-M11) or pCV0 (M12, control) plasmid DNA. The monkey M13 was a naive animal infected with 2.85 MID_{50} of SHIV89.6P.

5

FACS analysis of the percentage and absolute number of the CD4 and CD8 lymphocytes (Table 34) confirmed the virological data.

In fact, a significative decline of the CD4 was observed at 15 and 60 days post-challenge only for the naive monkey M13, resulted infected as indicated by the positiveness of plasma antigenemia, proviral DNA, and virus isolation. (Table 33).

TABLE 34

FACS analysis of CD4+ and CD8+ lymphocytes.

Monkey	Days post challenge with SHIV89.6P											
	0			15			30			60		
	% (cells/μl)			% (cells/μl)			% (cells/μl)			% (cells/μl)		
	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+
M9	21.5 (1500)	37.6 (2630)	0.57	ND	ND	ND	26.4 (1340)	51.6 (2610)	0.51	30.6 (2000)	45.5 (2980)	0.67
M10	39.5 (1050)	36.3 (960)	1.1	ND	ND	ND	34.8 (1730)	41.8 (2080)	0.83	31.6 (3760)	52.2 (6200)	0,61
M11	35.8 (1080)	37.7 (1140)	0.95	ND	ND	ND	28.7 (1330)	36.7 (1710)	0.78	24.5 (890)	48.7 (1770)	0.5
M12	30.9 (1860)	46 (2760)	0.67	ND	ND	ND	26.7 (1300)	49.6 (2420)	0.54	23.7 (2620)	52.1 (5760)	0,45
M13	40.5 (2590)	39.7 (2544)	1,01	38.4 (434)	33.6 (380)	1.14	35.1 (1721)	32.2 (1479)	1.16	3,1 (111)	62.3 (2225)	0.05

The assay was performed as described in Table 18. The macaques were injected i.m. with 1 mg of either pCVTat (M9-M11) or pCV0 (M12, control) plasmid DNA. The monkey M13 was a naive animal infected with 2.85 MID_{50} of SHIV89.6P.

These results demonstrate that the vaccination with the pCVTat plasmid DNA was well tolerated and non-toxic and confirm those data on the safety and tolerability of the DNA vaccination, obtained in the first pilot study. In addition, these data provide evidence that the pCVTat DNA plasmid induces a specific humoral (although weaker than that induced by the immunization with the Tat protein) and cellular anti-Tat response with antiviral effects, part of which may be due to particular sequences present in the pCV0 vector that could function as adjuvants. Immunization protocols that will include combinations of the DNA coding for other HIV-1 and/or cytokines genes described in the Example 3 will be evaluated. In these experiments SHIV containing the tat, rev, and nef genes of HIV will be used [Shibata et al., J. Virol.65:314 (1991); Li et al.. J. AIDS 5:639 (1992); Sakuragi et al., J. Gen. Virol. 73:2983 (1992); Li et al., J. AIDS 5:639 (1992); Igarashi et al., AIDS Res. Hum. Retrov. 10:1021 (1994); Luciw et al., Proc. Natl. Acad. Sci. 92:7490 (1995); Reimann et al., J. Virol. 70:6922 (1996)].

The pCV0 and pCVTat plasmid DNAs will be inoculated in the animals utilizing other delivery systems that may improve the immunization effectiveness, such as liposomes, nanoparticles, erythrocytes, gene gun delivery, or Tat DNA will be delivered through the utilization of herpes vectors as described in the prophetic Examples 9 and 10.

Example 6. Therapeutic vaccine.

A protocol of vaccination, based both on Tat-protein and Tat DNA, was made to evaluate the safety and toxicity of anti-Tat vaccine in already infected individuals. The experiment was performed on monkeys infected with decreasing doses of SHIV89.6P and with immunodeficiency disease (AIDS). The viral stock used for the infection was obtained from spleen and lymph nodes of a cynomolgus monkey infected 14 days before. Lymphocytes, purified by mechanical separation, were divided into two aliquots ($1,5\times10^6$ cells/ml each). One aliquot was depleted of CD8+ T lymphocytes by using immuno-magnetic beads (Dynal, Norway). Both cultures were stimulated with PHA (1 µg/ml) for three days and seeded at the concentration of 1×10^6 cells/ml in presence of 50U/ml of IL-2. Virus presence in the supernatants was detected by reverse transcriptase (RT) in the culture

medium harvested after three days by using 1 ml supernatant of the cell culture, clarified and ultracentrifuged at 100,000 rpm, for 11 minutes at +4°C (Beckman TL-100 ultracentrifuge) and the thus obtained pellet was lysed. Thirty µl were added to the reaction mix (TRIS HCl 1M, pH 8; $MgCl_2$, 0.5 M; KCl, 1M; Poly A 1mg/ml; oligo-dT 12-18 100 µg/ml; DTT 0.02 M; 1,2 $^{3}[H]$-Methyl thymidine tri-phosphate 1mCi/ml) and incubated at 37°C for 60 minutes. The reaction was stopped by adding 500 µl of Na Pyrophosphate 0.1 M pH5 and 600 µl of tri-chlor-acetic acid (TCA) 20% and the sample was spotted on a 0.45 µm filter (Millipore) and then read with a β-counter after the addition of 5 ml of scintillation cocktail (Filter Count, Packard).

Culture media containing more than 20,000 cpm were centrifuged and supplemented with 10% human serum AB. The virus was concentrated by ultra-centrifugation at 30,000 rpm (90 minutes at 4°C), resuspended in RPMI 1640 containing 10% of human serum (AB group) and then stored in liquid nitrogen. The viral stock was titred in vitro on the human cell lines CEMx174 and C8166 (3×10^3 $TCID_{50}$/cell), and in vivo on cynomolgus monkeys ($3.17 \times 10^{5.69}$ MID_{50}/ml).

A first pilot experiment has been performed on 7 monkeys infected i.v. with SHIV89.6P prepared as described above. Each monkey received 1 ml of SHIV diluted in saline buffer supplemented with 2% of human serum (AB, Rh-) according to the following protocol. One monkey (IM1) was inoculated with 1:500 of viral dilution; two monkeys (IM2, IM3) received the dilution 1:5,000; two monkeys (IM4, IM5) were inoculated with 1:50,000; the monkey IM6 received the 1:500,000 dilution; the last monkey (IM7) received 1:5,000,000 dilution. Each monkey was bleed (10 ml) at day 7 before infection with SHIV for determination of the basal parameters. Serum and plasma samples were frozen at –20°C or –80°C and then used to re-suspend the protein inoculum. At time 0 all monkeys were inoculated with SHIV89.6P. Monkeys were checked daily. Moreover, at day 0 and after 2 and 4 weeks they were bled and 10 ml of blood were used for hemato-chemical determinations (chemical-clinical analysis, electrolytes, white cells and platelets counts, hemoglobin) and virological and immunological analysis (i.e. plasma p27 Ag determination and viral load in plasma and cells). At week 4 pos

infection, 6 monkeys (IM1-6) were infected. The monkey IM7, which received the lowest viral dilution (1:5,000,000) was SHIV-negative (Table 35).

TABLE 35

Detection of the presence of SHIV89.6P in monkeys infected with serial viral dilutions

Monkey	SHIV 89.6P dilution	Weeks post infection					
		0		2		4	
		Viral isolation[a]	p27 (pg/ml)[b]	Viral isolation[a]	p27 (pg/ml)[b]	Viral isolation[a]	p27 (pg/ml)[b]
IM1	1:500	ND	ND	+	>450	+	47
IM2	1:5.000	ND	ND	+	>450	+	161.8
IM3	1:5.000	ND	ND	+	>450	+	6.67
IM4	1:50.000	ND	ND	+	<20	+	>450
IM5	1:50.000	ND	ND	+	>450	+	166.7
IM6	1:500.000	ND	ND	+	>450	+	0
IM7	1:5.000.000	ND	ND	-	0	-	0

[a]Virus isolation and [b] plasma p27 Ag (pg/ml) were carried out as described in the legend to table 17. Monkeys were inoculated i.v. with serial dilutions of the virus stock, as described in text.

After 7 weeks from infection, all the animals showing serious immunodeficiency symptoms were vaccinated with both the Tat protein and DNA of the plasmid pCVTat according to the following protocol. Monkeys IM1, IM3, IM5 and IM6 received the Tat protein (20μg), dissolved in 250 μl of PBS-A supplemented with 0,1% BSA and 20% of autologous plasma and then added to 250μl of Alum adjuvant. The protein inoculum was performed sub-cutaneously on a single site of monkey's upper back, whether the plasmid DNA pCVTat (1 mg), resuspended in 1 ml of PBS-A, was injected i.m. in a different site in the back. Monkeys IM2 and IM4 (controls) were injected with 250 μl of Alum and 250 μl of PBS-A, 0.1% BSA 20% autologous plasma, s.c., in a site of the upper back and with pCV-0 plasmid DNA (1 mg) resuspended in 1 ml of PBS-A, i.m., in a site in the upper back different from the previous one. The uninfected monkey IM7 was not vaccinated. The schedule of vaccination consisted of a time 0, corresponding to 7 weeks after SHIV infection, and 1, 4, 5, 10, 11, 13, 14, 17, 18 weeks on. To evaluate the

effects of this vaccination on disease progression, each macaque was daily checked for the presence or signs of disease and at time 0 and after 3, 8, 12, 18 and 21 weeks, 10 ml of blood were withdrawn for laboratory tests (chemical clinical analysis, electrolytes, white cells and platelets counts, hemoglobin), for the evaluation of immunological status (presence of specific immunoglobulins, measure of Th1 and Th2 cytokines, chemokines production), for characterization of lymphocytes by FACS analysis (CD4, CD8, CD28, CD40, CD86, CD20, CD2, CD26 and CD20), and finally for evaluation of virological parameters (proviral DNA detection by semi-quantitative PCR, plasma viral load by competitive RT-PCR, plasma p27 Gag antigen by ELISA and presence of anti-SHIV Ab, as described previously). Other boosts will be made on the basis of the immunological, virological and clinical results.

After the last inoculum, monitoring will be scheduled monthly and at the appearance of clinical modifications. PBMC, sera, plasma and urine samples will be frozen at every time point for future tests as previously described.

The results already available from this experiment, obtained at week 8 after immunization, are described. In both the vaccinated asymptomatic and control monkeys no signs of inflammation and neo-angiogenesis in the inoculation sites or general symptoms of disease were observed. No modifications of the clinical status were evident in the monkeys already symptomatic. Moreover, no activation of viral replication was detected. Taken together these results indicate the absence of toxicity or increased viral replication in the monkeys vaccinated (Table 36).

TABLE 36

Analysis of virological parameters

Monkey	Weeks from the beginning of vaccination					
	0		3		8	
	p27 (pg/ml)	DNA PCR copies/μg	p27 (pg/ml)	DNA PCR Copies/μg	p27 (pg/ml)	DNA PCR Copies/μg
IM1	12.3	68	17.3	52	141	41
IM3	0	61	0	48	0	71
IM5	97.1	20	21.7	15	23.6	95
IM6	0	43	0	55	0	24
IM2	21.2	ND	36.6	53	27.4	78
IM4	81	195	22	288	15.4	135
IM7	ND	ND	ND	ND	0	>1

The tests were performed as described in Table 17. Monkeys IM1, IM3, IM5 and IM6 were injected with Tat protein (20μg) and Alum adjuvant s.c. and with pCVTat (1mg) i.m.. Monkeys IM2 and IM4 (infected controls) were injected with Alum adjuvant s.c. and pCV0 (1mg) i.m.. IM7 was an uninfected naive monkey.

FACS analyses indicate that no modifications were observed in CD4+ and CD8+ lymphocytes after vaccination (Table 37).

TABLE 37

FACS analysis of CD4+ and CD8+ lymphocytes

Monkey	Time 0			Weeks from beginning of vaccination								
				0			3			8		
	% (cells/µl)			% (cells/µl)			% (cells/µl)			% (cells/µl)		
	CD4+	CD8+	CD4/CD8	CD4+	CD8+	CD4/CD8	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8
IM1	25,39 (1264)	36.8 (1831)	0.69	3,3 (101)	64.16 (1963)	0.05	2,32 (52)	63,34 1431)	0,04	3.41 (96)	55,41 (1559)	0,06
IM3	19.26 (869)	26,45 (1193)	0.73	2.84 (74)	58.22 (1526)	0,05	3,21 (92)	58.16 (1663)	0.05	3.18 (91)	50,12 (1434)	0,06
IM5	24.75 (580)	58.04 (1361)	0.42	2.28 (38)	57.3 (946)	0.04	2,89 (48)	55,6 (917)	0,05	2,15 (60)	54,3 (1527)	0,04
IM6	40,46 (2590)	39.74 (2544)	1,01	3.12 (111)	62.3 (2225)	0.05	2.75 (138)	65,40 (3290)	0,04	2,3 (73)	52,18 (1659)	0,04
IM2	42 (1787)	34,7 (1476)	1.21	2,41 (68)	58.12 (1632)	0,03	2,7 (121)	57.6 (2580)	0,05	1,89 (66)	50,6 (1763)	0,04
IM4	30,72 (1589)	27.76 (1680)	1,10	2,12 (113)	61,13 (3248)	0,03	1.92 (90)	60.3 (2828)	0,03	3,12 (164)	53,12 (2790)	0,06
IM7	17.02 (871)	55.8 (2857)	0,30	ND	ND	ND	20.26 (770)	51.40 (1957)	0,39	24,1 (868)	50,43 (1842)	0,48

FACS analysis was performed as described in the legend to Table 18. Monkeys IM1, IM3, IM5 and IM6 were injected with Tat protein (20µg) and Alum adjuvant s.c. and with pCVTat (1mg) i.m.. Monkeys IM2 and IM4 (controls) were injected with Alum adjuvant s.c. and pCV0 (1mg) i.m.. IM7 was an uninfected naive monkey.

These data confirm that both Tat protein and pCVTat plasmid DNA, at the used doses and inoculation routes, were well tolerated and without any toxic effect in the vaccinated monkeys and, moreover, they did not increase viral replication in infected animals.

Example 7.

Co-stimulation of purified CD4+ lymphocytes from SIV-infected monkeys, with anti-CD3/28 coated beads results in a logarithmic expansion of the cell number without significant viral replication and transmission.

Peripheral blood cells were depleted of CD8+ sub-cell population by using anti-CD8 paramagnetic beads (Dynal, Oslo; Dynabeads M-450 CD8). The purification degree was evaluated by FACS analysis and considered as acceptable if higher than 95%. The CD8-depleted cells (named CD8⁻PBMC) were grown in the presence of PHA (2μg/ml) and IL-2 (40U/ml) or immuno-magnetic beads previously coated with two monoclonal antibodies against the CD3 (Clone FN18, BioSource) and the CD28 (Clone 9.3) antigens (anti-CD3/28 beads). To improve the binding of anti-CD3/28 beads with target cells, the incubation was performed on a rotating wheel disposal. Then, the bound cells (named CD8-CD3+CD28+) were selected with a magnet and seeded in culture. Three times a week, cell concentrations were adjusted to the starting level and IL-2 was added where indicated; moreover, regarding the cells stimulated with anti-CD3/28 beads, preliminary results suggest that the continuous stimulation regimen coupled with a constant control of the bead:cell ratio, adjusted at every time point, is highly effective. Our previous studies have shown that in the absence of exogenous IL-2, the CD8-CD3+CD28+ cell population proliferates better then CD8-PBMC stimulated with anti-CD3/28 beads. Moreover, the addition of exogenous IL-2 (40U/ml, three times per week) significantly increases the kinetic of proliferation both in terms of number of cells and duration of effect (Figure 14).

To evaluate the antiviral activity of this stimulation, CD8-CD3+CD28+ purified cells from 4 uninfected monkeys were infected at day 0 with 0.1 M.O.I. of SIV and then cultivated under continuous stimulation. CD8-PBMC stimulated with PHA and IL-2 were the control of the experiment. Viral infection was followed through

detection of p27 Gag viral antigen in culture supernatant by a commercial ELISA (Coulter, Hialeah, FL). The p27 levels (ng/ml) were measured on day 6 and 12 after infection. As shown in figure 15, there is a significant difference in the infection in the two stimulation regimens in terms of infection. In fact, at day 6 after infection, the p27 antigen in the CD3/28 beads-stimulated cultures was 40% to 87% lower than cultures stimulated with PHA plus IL-2, and at day 12 this difference was increased in 2 out of 4 monkeys. This suggests a reduction of viral infection susceptibility. In only one case (MK 9401) we observed a viral propagation also in stimulation with anti-CD3/28 beads.

The results here described demonstrate that *Macaca fascicularis* is a good model for the ex vivo expansion of lymphocyte sub-population by anti-CD3/28 beads co-stimulation, without viral replication. This represents the rationale for the therapeutic vaccine we propose, based on expansion and re-infusion of autologous anti-viral specific lymphocytes, in HIV-infected individuals.

Example 8.

Use of dendritic cells for vaccination.

The dendritic cells (DC) and macrophages, in a lesser extent, are able to efficaciously present antigens Ag to the T lymphocytes and induce proliferation or acquisition of specific cytotoxic activities. These cells are named "antigen presenting cells"(APCs) and can start the immune response. Thus, DC may be utilized in *ex vivo* immunization protocols. For this reason, DC precursors were isolated from peripheral blood of *Macaca fascicularis* by culturing in vitro adherent cells after seven days of GM-CSF and IL-2 stimulation. Alternatively, CD34+ cells were purified with immuno-magnetic beads and then cultured in vitro with GM-CSF and TNF-α for 14 days. To confirm that DC were isolated, morphologic analysis and phenotypic characterization (FACS analysis and immuno-histochemistry) were performed. Functional analysis was based on the unique capability of DC to induce proliferation of allogeneic lymphocytes.

The results obtained fully confirm the effectiveness of the purification and the functional characterization of DC. Conversely, phenotypization is partial owing to the cross-reactivity of the monoclonal antibodies directed against the human

linear markers with the monkeys ones. In details, to isolate DC precursors, PBMCs, obtained by Ficoll density gradient centrifugation, were again stratified on Percoll discontinuous gradient (50% and 42.5%). The cellular fraction that, after centrifugation at 500 g for 30 min, was between the two gradients was mainly constituted of monocytes (as confirmed by FACS analysis, data not shown). These cells were kept at 4°C to avoid cell adhesion to the plastic tubes, then collected, washed, counted and seeded in culture at 37°C. The day after, non adherent cells were washed away with 4 gentle washings. To induce differentiation into DC, a complete medium supplemented with GM-CSF (200 ng/ml, Leucomax, Sandoz, Milan, Italy) and IL-4 (200 U/ml, Pepro tech, London, England) was added to adherent cells. As control, a complete medium without cytokines was added to induce the normal differentiation of monocytes in the macrophage lineage. Every 4-4 days, half of the supernatant was replaced with fresh medium identical as that one utilized at day 0. The maturation of DC in the wells treated with cytokines was detected by typical morphological changes, like clustering, loss of adherence and development of cellular offshoots. The monocyte/macrophages adherent cells grown without cytokines were detached by EDTA treatment (0,5 mM in PBS-A without calcium and magnesium), washed twice, counted and resuspended in fresh medium at different concentrations depending on the experiment performed. For the allogeneic mixed leukocyte reactions (AMLR), the obtained APCs (DC or macrophages) were tested with a fixed amount of allogeneic T lymphocytes, purified by Ficoll and Percoll gradients and adhesion, and then frozen. The AMLR was performed in 48-well plates with 0.5×10^6 T lymphocytes and serial dilutions of APCs. At day 4 of culture, a fixed amount of the cell suspension was seeded in a 96-well plate in triplicate. One μCi of ^{3}H-tymidine was added to each well and the plate was then incubated for 16 hours. At the end of incubation, the amount of ^{3}H-tymidine incorporated by the cells was measured with a β-counter and expressed as counts per minute (cpm). Results indicate that the DC obtained are potent APC as demonstrated by the higher induction of proliferation in allogeneic human lymphocytes compared to the macrophages stimulation (Figure 16A), and by the capability to induce T

lymphocyte proliferation in monkeys at all the concentrations used (Figure 16B). For the use in vaccination, DC will be resuspended at the concentration of 1×10⁶ cells/100 µl in RPMI 1640 supplemented with 5% of autologous serum, 10 mM of Hepes buffer, 100 U/ml of penicillin-streptomycin, 0.5 mg/ml of amphotericin B and 0.03% of glutamine, and then incubated for 2 hours at 37°C in presence of Tat protein or Tat-peptides or combination of Tat, Rev, Nef, Gag and/or cytokines. Then, this treated DC will be inoculated twice or more within 2-4 weeks from first injection, intravenously. Alternatively, DC will be transduced with *tat*-gene-containing vectors alone or associated with other vectors mentioned above and then injected intravenously.".

Note 68: page 59 of the description: line 1: after the word "gordonii" erase "host in the oral cavity in humans)" and insert "and Lactobacillus)".

Note 69: page 59 of the description: line 7: after the word "Vaccine" erase "1997, in press] and insert "15:1330 (1997); Pozzi et al., in "Gram-positive bacteria as vaccine vehicles for mucosal immunization", eds. Pozzi G. & Wells, J.M. – Landes, Austin, p. 35 (1997); Oggioni, et al., Gene 169:85 (1996); Rush, et al., in "Gram-positive bacteria as vaccine vehicles for mucosal immunization", eds. Pozzi G. & Wells, J.M. – Landes, Austin, p. 107 (1997); Medaglini et al., Biotech. Annu. Rev. 3:297 (1997); Medaglini et al., Am. J. Reprod. Immunol. 39:199 (1998)]. These bacteria can work as live vectors of vaccines and take the advantage to cause a prolonged stimulation of the immune system. Moreover, we will evaluate the possibility to co-express, on the bacterial surface, viral Ag and molecules involved in the immune response, such as the B sub-unit of the temperature-sensitive toxin of *E. Coli* or cytokines. The preparation of the recombinant strains of *S. Gordonii* will be carried out as previously described [Oggioni et al., Vaccine 13:775 (1995)]. Briefly, (i) chromosomal integration of recombinant DNA molecules; (ii) transcriptional fusions with strong chromosomal promoters; (iii) transcriptional fusions with the gene coding for the protein M6, a surface protein of Streptococcus. The recombinant strains of *S. Gordonii* will be utilized to colonize the vaginal mucosa of the monkeys. It has been demonstrated that the recombinant strains of *S. Gordonii* which express the V3 region of gp120

of HIV-1 and the E7 protein of HPV-16, permanently colonize the vaginal mucosa of the mouse after a single inoculum, inducing an Ag-specific antibody response both local and systemic. The systemic response is in prevalence composed of IgG2a antibodies, which suggests a Th1-type response [Medaglini et al., Biotech. Annu. Rev. 3:297 (1997); Medaglini et al., Vaccine 15:1330 (1997)]. We will select human vaginal strains of Lactobacillus, which are able to colonize the vaginal mucosa of the monkeys. Thereafter, an already developed genetic system will be utilized, which permits the expression of heterologous Ag on the surface of Lactobacillus (Rush, 1997). This strategy is based on: (i) cloning of genetic fusions (*emm6*/heterologous gene) into insertion vectors which carry homologies with the conjugative transposon Tn916; (ii) transformation of the vectors in bacterial strains which work as intermediate host (*Bacillus Subtilis*); (iii) conjugative mobilization of the recombinant transposons from *B.subtilis* to *Lactobacillus* strains. The recombinant strains of *Lactobacillus* will be utilized to colonize the vaginal mucosa of the monkeys.

Vaginal samples will be obtained utilizing special absorbent filters [Di Fabio et al., submitted; Medaglini et al., Biotech. Annu. Rev. 3:297 (1997); Medaglini et al., Vaccine 15:1330 (1997)]. Colonization will be evaluated by plating the vaginal samples on selective plates and expression of HIV Ag in vivo will be monitored by immuno-fluorescence on vaginal swaps [Medaglini et al., Biotech. Annu. Rev. 3:297 (1997)]. By using already standardized methods [[Di Fabio et al., submitted], the vaginal swaps will be utilized for i) Papanicolau test, in the case of vaginal vaccination; ii) presence of vaccine antigens in the cells; iii) phenotypic characterization of cells by cytofluorometric analysis (CD1, CD2, CD4, CD5, CD8, CD11c, CD14, CD20, CD28, CD40, CD25, HLA-DR); iv) evaluation of cytokine expression (IL-2, IFNγ, TNFα, IL-4, IL-10, IL-15, semi-quantitative RT-PCR), determination of the presence of cytokines and β-chemokines in the mucosal fluids, by Elisa assays; v) dosage of total and specific immunoglobulins (IgA and IgG) in the mucosal fluid by Elisa [Di Fabio et al., Vaccine 15: 1 (1997)]. One month after the last inoculum of the immunogen, the monkeys will be infected intravenously or through the mucosal route with the SHIV 89.6P. The follow-up of

the monkeys will be carried out as described in the Example 4. Blood samples will be obtained in order to execute the routine laboratory exams, the evaluation of immunological parameters, both humoral and cellular, as described in the Example 4.".

Note 70: page 59 of the description: line 9: after the word "monkeys" erase "cynomolgus"."

Note 71: page 59 of the description: line 11: after the word "induced" erase "with the proteic immunogens above described," and insert "by administering the protein immunogens, above described, directly through the mucosal route in the presence of adjuvants, such as the thermo sensitive toxin of E. Coli and the choleric toxin, or".

Note 72: page 59 of the description: after the last line, go ao a new line and insert: "Moreover, the inventor believes that recombinant herpes vectors, expressing the above described viral proteins, can be excellent systems to induce an effective mucosal immune response. Recombinant viral vectors from the herpes simplex type 1 virus (HSV-1) will be utilized to express viral proteins for the induction, of a systemic (through cutaneous immunization, i.d.) and mucosal (through the oral, vaginal or nasal route) responses. Non pathogenic, non replicative herpes vectors will be utilized [Marconi et al., Proc. Natl. Acad. Sci 93:11319 (1996)] for their ability to include large exogenous sequences, without interfering with the efficacy of the infection [Glorioso et al., Ann. Rev. Microbiol. 49:675 (1995); Huard et al. Gene Ther. 2:385 (1995)]. Therefore, vectors able to contain more than one HIV gene (accessory, regulatory and structural) will be constructed. The mucosal immunity could be induced by an oral, vaginal or nasal vaccine. The herpes vectors can be used in these vaccinal approaches, since HSV-1 can be administered directly by the mucosal route [Bowen et al., Res. Virol. 143:269 (1992); Kuklin et al., J. Virol. 240:245 (1998)]. The recombinant viruses will be constructed utilizing a two-steps method which facilitates the insertion of exogenous sequences into the viral genome. The first step requires the insertion of an expression cassette with a reporter gene (β-galactosidase LacZ) cloned in the restriction site PacI, which is not present in the HS

genome, flanked by the wanted target sequence of HSV-1, using the standard procedure for the homologous recombination, to interrupt the HSV-1 gene. The recombinant virus is selected by formation of plaques with a blue phenotype, using "x-gal staining". The digestion of viral DNA with PacI releases the marker gene and generates two large fragments of viral DNA, not able to produce infectious viral particles. The second step consists of a co-transfection of the viral DNA, digested with the same plasmid used to create the deletion, where the reporter gene is substituted by the wanted gene. The recombinant viruses will be identified through the selection of plaques with a white phenotype after "x-gal staining". This recombination will lead to the elimination of PacI sites allowing the use of this method to insert many genes in different loci of HSV-1 genome [Krisky et al., Gene Ther. 4:1120 (1997)]. By crossing the different vectors containing the single genes, we might be able to create all the different genetic combinations. The vector containing all the wanted genes will be isolated by screening with different markers, phenotypes and selective growth on competent cells. All the combinations will be created by alternating DNA transfections and viral recombinations.

Vectors expressing the single genes tat, rev, nef or gag, will be constructed utilizing, as basic vector, that one containing the mutations in the genes 4-/22-/27-/41-, that is better for the low toxicity and the strong expression of the exogenous gene, compared to the other HSV-1 not replicative vectors. Constitutive promoters will be used, such as those from HCMV (human cytomegalovirus immediate early promoter), or as ICP0 Iep (infected cell protein immediate early promoter) and the Moloney Murine Leukemia virus LTR, for inducing the expression of the genes above mentioned. Non replicative HSV-1 vectors expressing HIV-proteins in different combinations will be constructed. The production of these viruses containing more different genes will be obtained by a genetic crossing over of the vectors containing the single genes described in the previous point. Double, triple and quadruple vectors will be created. The vectors will be inoculated in the monkeys i.d. or through mucosal (oral, vaginal or nasal) route with particular attention to this last type of administration [Bowen et al., Res. Virol. 143:269

(1992); McLean et al., J. Infect. Dis. 66:341 (1994); McLean et al., Vaccine 14:987 (1996)]. Vaccination schedule consists of multiple inocula at different time points, which must be determined in relation to immunogen or the combination of immunogens. During immunization the animals will be monitored for the evaluation of hematochemical and immunological parameters as described in the Example 4. With methods already standardized (Di Fabio et al., submitted) vaginal samples will be obtained, that will be studied as previously described in this Example.

Prophetic example 10

Delivery systems

Tat (protein and/or DNA) alone or in combination (as described above) will be inoculated using new delivery systems, such as erythrocytes or nanoparticles.

The delivery system involving the use of erythrocytes is based on the possibility to deliver the antigen bound on the membrane of autologous erythrocytes. In fact erythrocytes, at the end of their life span (around 120 days in humans), are removed from the circulation by the macrophages, known to have the function of professional antigen presenting cells. This property can be used for vaccine strategies. Thus, antigens will be bound to the membrane of the erythrocytes with a particular technique [Magnani et al., Biotech. Appl. Biochem. 16: 188 (1992); Magnani et al., Biotech. Appl. Biochem. 20: 335 (1994)], that allows the preservation of the immunogenic properties of the antigen [Chiarantini et al., Vaccine 15: 276 (1997); Chiarantini e at., Clin. Diag. Lab. Immunol. 5: 235 (1998)]. Through this procedure, biotinilation of erythrocytes can be performed in the absence of significative modification of their properties and life span [Magnani et al., Biotech. Appl. Biochem. 16: 188 (1992)]. Biotinilated erythrocytes can act as carriers for immunogens when these ones are suitably biotinilated. So modified erythrocytesregularly circulate untill they are removed by the macrophagic system that presents the antigen, thus inducing an immune response. The resulting production of antibodies will opsonize the erythrocytes which bind the antigen, thus accelerating the removal from circulation. The main advantages of such method are related to the reduced amount of antigen needed to induce a humoral

and cellular response to the possibility of a long lasting immunization due to the lasting presence of antigens carried by the erythrocytes and to an adjuvant functions provided by the system itself. In fact, it has been shown in animal studies that the administration of antigens bound on the membrane of autologous erythrocytes induces a similar or higher immune response compared to the immune response obtained with the same antigen administered with Freund's adjuvant [Chiarantini et al., Vaccine 15: 276 (1997)]. These properties are very useful to develop an anti-HIV vaccine, in particular when it is needed to increase the immunogenicity of the antigen, when the antigen availability is a limiting factor and in particular when a low number of immunizations is required. In addition, this strategy can be used when no adjuvants are included in the vaccination protocol. In fact, it has been shown in the murine model that antigens administered through autologous erythrocytes induce similar or higher immune responses compared to those obtained with the same antigen administered with Freund's adjuvant known as the most powerful adjuvant commercially available [Chiarantini et al., Vaccine 15: 276 (1997)], although not approved for human studies because of the important side effects. Thus, the adjuvant effect of erythrocytes carrying Tat protein, alone or in combination with other immunogens previously described, will be analyzed in non human primates. Comparison among these data and those obtained with the administration of Tat protein in the presence of Alum, RIBI or ISCOM will be performed.

The use of nanoparticles can represent an additional delivery strategy. Functional nanoparticles represent an important system for the transport and release of proteins and DNA [Chavany et al., Phar. Res. 9: 441 (1994); Zobel et al., Antisense Nucleic Acid Drug Dev. 7: 483 (1997)] in that they can be produced with a high affinity for these biologically active molecules.The nanospheres are colloid polymeric particles of different chemical composition, with a large range of diameter from 10 to1000 nm. It is possible to adsorb different kind of substances on the surface or inside the nanospheres (oligonucleotides, drugs, proteins, peptides, DNA) that are then brought to the cytoplasm or to the nucleus of cells where they are slowly released. In addition, a small amount of the immunogen is

needed to be delivered due to the characteristics of nanospheres. Nanoparticles are a good delivery system especially for molecules with low stability in the intracellular environment or when the delivery is directed to a specific target cell. The inventor believes that nanospheres can be used to deliver the viral antigen above described. It is possible to prepare and characterize three types of nanospheres designed for the delivery and controlled release of DNA (nanospheres type 1 and 2) and proteins (nanospheres type 3).

For the DNA delivery, two types of nanospheres (nanospheres type 1 and 2) are available. The first type of nanospheres (nanospheres type 1) has a triple layers structure with an external layer of poly-oxy-ethylen-glicole (PEG). Recent reports based on stealth systems studies [Allen et al., Biochim. Biophis. Acta 1237:99 (1995); Lasic et al., Chemical Reviews 95: 2601 (1995)], show that PEG makes nanospheres invisible to Kupfer cells. The more internal layer is made of monomers with tensioactive features containing quaternary ammonium groups that reversibly adsorb the DNA through a mechanism of ionic exchange and an internal core made of methyl-metacrylate as monomer. These nanospheres are obtained by polymerization in microemulsion involving the polymerization of a vinilic or vinilidenic monomer in the presence of a mix of tensioactive reagents. These reagents are thus able to polymerize the monomer. Of these, one has a quaternary ammonium group interacting with oligonucleotides and the other one has a long chain of PEG (M. Laus, It. Pat. BO97000641).

The second type of DNA delivery system is made of functional nano and microspheres (nanospheres type 2) with hydrogel characteristics. These nanospheres should be made in the presence of a DNA solution to trap it inside the delivery system.

Nanospheres core-shell are needed to deliver proteins (nanospheres type 3). They are made by an internal core of poly-methyl-metacrylate and an external shell of hydrosoluble statistic copolymer of acrylic acid and methyl-metacrylate, known to have an high degree of affinity for proteins [Laus et al., Polymer 37: 343 (1996); Laus et al., Polymers for Adv. Techn. 7: 548 (1996)]. This copolymer is commercially available (EUDRAGIT) and is obtained with different percentages

the two co-monomers. The preparation process leading to the manufacture of this second type of nanospheres involves the polymerization in dispersion. The synthesis involves the radical polymerization of a vinilic or vinilidenic monomer in the presence of EUDRAGIT having steric stabilizing functions. After nanospheres nucleation, the EUDRAGIT arranges outside the particles. Thus, modifying the concentration of the radical initiator, the ratio between the monomer and EUDRAGIT and the reaction time, numerous nanospheres samples are obtained with different morphologic and chemical characteristics.

Thus, it can be evaluated whether the delivery of Tat protein or Tat DNA by nanoparticles, alone or in combination with the immunogens mentioned above (either protein or DNA) will induce an immune response against HIV. In particular, the humoral or cellular-mediated immune responses will be evaluated and compared (intensity and extension) to those obtained with the not delivered immunogens in the monkey model.

The inventor believes that the information derived from these studies can be useful to develop an anti-HIV vaccine. In addition, the information derived from this experimental protocol will be transferred also to other vaccines studies, in particular to those studies dealing with low immunogenicity recombinant proteins or peptides. The possibility to develop a vaccine with only one administration will lead to enormous advantages in terms of efficacy of the vaccine and decrease of managing costs of vaccine programs.

Note 73: pages 60 and 61 of the description: erase all the claims and insert: "

1. Protein or peptide or DNA vaccine, prophylactic and/or therapeutic, against AIDS, tumors, syndromes and symptoms associated with the HIV infection, comprising wild-type Tat in its active form and/or its mutants and/or portion of the protein or peptides or a DNA encoding for said proteins or said portions or peptides.
2. Vaccine according to claim 1 in which Tat has the following nucleotide sequence (Seq.1) :

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTC
AGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTG

CCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAA
GCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTT
CTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAGGCC
AAGGAATAG 3'

and any other Tat variant of any HIV type and subtype.

3. Vaccine according to claim 1 in which Tat has the following amino acid sequence :

NH2-EPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKAISY
GRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

and any other Tat variant of any HIV type and subtype.

4. Vaccine according to claim 1 in which mutants are selected among the ones having the following nucleotide sequences or part of them:

Nucleotide sequence of cys22 mutant (Seq.2)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCGGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATT
GCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAG
AAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAG
TTTCTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAGG
CCCGAAGGAATAG 3'

Nucleotide sequence of lys41 (Seq.3)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAG
GCCCGAAGGAATAG 3'

Nucleotide sequence of RGDΔ mutant (Seq.4)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCA

GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCCGACAGGCCCGAAGGA
ATAG 3'

Nucleotide sequence of lys41-RGDΔ mutant (Seq.5)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCCGACAGGCCCGAAGGA
ATAG 3'

5. Vaccine according to claim 1 in which mutants are selected among the ones having the following amino acid sequence or part of them:

Amino acid sequence of cys22 mutant

NH2-MEPVDPRLEPWKHPGSQPKTAGTNCYCKKCCFHCQVCFITKA
LGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Amino acid sequence of lys41

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Amino acid sequence of RGDΔ mutant

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Amino acid sequence of lys41-RGDΔ mutant

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

6. Vaccine according to claim 1 in which the Tat portions are selected among the peptide sequences

Pep. 1. MEPVDPRLEPWKHPGSQPKT

Pep. 2. ACTNCYCKKCCFHCQVCFIT

Pep. 3. QVCFITKALGISYGRK

Pep. 4. SYGRKKRRQRRRPPQ

Pep. 5. RPPQGSQTHQVSLSKQ

Pep. 6. HQVSLSKQPTSQSRGD

Pep. 7. PTSQSRGDPTGPKE

7. Vaccine according to claims 1-6 comprising proteins or peptides conjugated with the T-helper universal epitope of Tetanus toxoid or any T-helper peptides.
8. Vaccine according to claims 1-7 in combination with recombinant proteins or peptides of HIV Nef, Rev or Gag or part of them.
9. Vaccine according to claim 1 comprising fusion proteins Tat (wild type or its mutants)/Nef, Tat (wild type or its mutants)/Rev, Tat (wild type or its mutants)/Gag or part of them.
10. Vaccine according to claim 1-9 in combination with recombinant immuno-modulant cytokines or other molecules, or part of them, augmenting antiviral immune response.
11. Vaccine according to claim 1 in which cytokines are IL-12 and/or IL-15.
12. Vaccine according to claim 1 comprising fusion proteins Tat(wild type or its mutants)/immuno-modulant cytokines, Tat (wild type or its mutants)/IL-12, Tat (wild type or its mutants)/IL-15, Tat (wild type or its mutants)/other molecules, or part of them, augmenting the antiviral immune response.
13. DNA vaccine according to claims 1, 2, 4 comprising DNA encoding for Tat wild-type or its mutants or part of them, inserted in expression vectors.
14. DNA vaccine according to claims 1, 2, 4 in combination with an expression vector including HIV rev, nef and gag genes, or part of them.
15. DNA vaccine according to claims 13 or 14 in which the vector is a plasmid co-expressing tat (wild-type or its mutants)/rev, tat (wild-type or its mutants)/nef, tat (wild-type or its mutants)/gag or part of them.
16. DNA vaccine according to claims 1, 2, 4 in combination with DNA molecules inserted in expression vectors encoding for immuno-modulant cytokines or other immuno-modulant molecules, or part of them, augmenting the antiviral immune response.
17. DNA vaccine according to claim 16 in which the cytokine is IL-12 and/or IL-15.
18. DNA vaccine according to claims 16 or 17 in which the vector is a plasmid co-expressing tat (wild-type or its mutants)/IL-12, tat (wild-type or its mutants)/IL-15, tat (wild-type or its mutants)/other molecules, or part of them, able to

augment the antiviral immune response.

19. Vaccine according to claims 13-18 in which the vector is pCV0.

20. Vaccine according to the previous claims including autologous dendritic cells treated and/or untreated according to the previous claims.

21. Vaccine according to the previous claims including adjuvants able to augment the antiviral immune response.

22. Vaccine according to claim 21 in which the adjuvant is selected among Alum, ISCOM, RIBI and mixtures thereof.

23. Vaccine according to previous claims comprising systems for delivery.

24. Vaccine according to claim 23 in which the systems for delivery are selected among nanoparticles, herpes vectors, red cells, bacteria and combinations thereof.

25. Vaccine according to claim 24 in which bacteria are selected among *Streptococcus gordonii* and *Lactobacillus*.

26. Vaccine according to claims 24 and 25 in which bacteria are modified to express viral antigens.

27. Vaccine according to the previous claims for the immunization of peripheral blood cells from infected individuals, expanded by co-stimulation with magnetic beads coated with anti-CD3 and anti-CD28 antibodies.

28. Therapeutic vaccine according to the previous claims, combined with inhibitors of viral replication.

29. Vaccine according to the previous claims in which the active principle is delivered to the mucosa (nasal, oral, vaginal or rectal).

30. Vaccine according to the previous claims in which the active principle is administered through systemic or local route.

31. Vaccine according to claim 30 in which the active principle is administered through intramuscular, subcute or intradermal route.

32. Vaccine according to claims 29-31 in which the active principle is administered in autologous serum

33. Tat nucleotide sequence (Seq. 1):

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT

CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAG
GCCCGAAGGAATAG 3'

34. Tat amino acid sequence

NH2-EPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKAISY
GRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

35. Tat mutant protein having nucleotide sequence selected among:

Nucleotide sequence of the cys22 mutant (Seq.2)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCGGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATT
GCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAG
AAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAG
TTTCTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAGG
CCCGAAGGAATAG 3'

Nucleotide sequence of the lys41 mutant (Seq.3)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCGAGGGGACCCGACAG
GCCCGAAGGAATAG 3'

Nucleotide sequence of the RGDΔ mutant (Seq.4)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCCGACAGGCCCGAAGGA
ATAG 3'

Nucleotide sequence of the lys41-RGDΔ mutant (Seq.5)

5'ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGT
CAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT
TGCCAAGTTTGTTTCATAACAAACGCCTTAGGCATCTCCTATGGCAGGAA
GAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAA
GTTTCTCTATCAAAGCAGCCCACCTCCCAATCCCCGACAGGCCCGAAGGA
ATAG 3'

36. Tat mutants amino acid sequence selected among:

Amino acid sequence of cys22 mutant

NH2-MEPVDPRLEPWKHPGSQPKTAGTNCYCKKCCFHCQVCFITKA
LGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Amino acid sequence of lys41 mutant

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE-COOH

Amino acid sequence of RGDΔ mutant

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

Amino acid sequence of lys41-RGDΔ mutant

NH2-MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITTAL
GISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSPTGPKE-COOH

37. Tat mutants with peptide sequence selected among :

Pep. 1. MEPVDPRLEPWKHPGSQPKT
Pep. 2. ACTNCYCKKCCFHCQVCFIT
Pep. 3. QVCFITKALGISYGRK
Pep. 4. SYGRKKRRQRRRPPQ
Pep. 5. RPPQGSQTHQVSLSKQ
Pep. 6. HQVSLSKQPTSQSRGD
Pep. 7. PTSQSRGDPTGPKE

38. Expression vector comprising a DNA sequence selected among the ones listed in claims 33 and 35 or parts thereof

39. Expression vector pCV0 including a DNA sequence selected among the ones listed in claims 33 and 35 or parts thereof.

40. Expression vector pCV0 comprising a cDNA sequence codifying for a gene selected among tat, rev, nef, gag, IL-12, IL-15 and combinations thereof.

41. Transformed cells including the vector according to claims 38-40.

42. Dendritic cells inoculated with Tat protein or its peptides or mutants or combinations with Rev, Nef, and Gag proteins and/or cytokines.

43. Dendritic cells transduced with an expression vector comprising tat gene.

44. Process for producing Tat protein or its mutants or its recombinant forms or parts of them comprising to cultivate the cells according to claim 41 and to isolate and purify the thus obtained protein or parts of it.

45. Process for preparing the pCV0 vector in which the corresponding cDNA according to claim 40 is amplified by PCR technique using primers selected among:

Seq. P1. Primer forward Rev: 5'ATGGCAGGAAGAAGC3'

Seq. P2. Primer reverse Rev: 5'CTATTCTTTAGTTCC3'

Seq. P3. Primer forward Nef: 5'ATGGGTGGCAAGTGG3'

Seq. P4. Primer reverse Nef: 5'TCAGCAGTCCTTGTA3'

Seq. P5. Primer forward Gag: 5'ATGGGTGCGAGAGCG3'

Seq. P6. Primer reverse Gag: 5'TTATTGTGACGAGGG3'

Seq. P7. Primer forward IL-12: 5'ATGTGGCCCCCTGGG3'

Seq. P8. Primer reverse IL-12: 5'TTAGGAAGCATTCAG3'

Seq. P9. Primer forward IL-15: 5'ATGAGAATTTCGAAA3'

Seq. P10. Primer reverse IL-15: 5'TCAAGAAGTGTTGAT3'

Seq. P11.Primer forward Tat: 5'ATGGAGCCAGTAGAT3'

Seq. P12. Primer reverse Tat: 5'CTATTCCTTCGGGCC3'

Seq. P13. Primer forward Tat/Rev: 5'GGCCCGAAGGAAATGGCA GGAAGAAGC3'

Seq. P14. Primer forward Tat/Nef: 5' GGCCCGAAGGAAATGGGT GGCAAGTGG3'

Seq. P15. Primer forward Tat/Gag: 5' GGCCCGAAGGAAATGGGT GCGAGAGCG3'

Seq. P16. Primer forward Tat/IL-12: 5' GGCCCGAAGGAAATGTGGC

CCCCTGGG3'

Seq. P17. Primer forward Tat/IL-15: 5' GGCCCGAAGGAAATGAGAAT TTCGAAA3'

46. Primer selected among:

Seq. P1. Primer forward Rev: 5'ATGGCAGGAAGAAGC3'

Seq. P2. Primer reverse Rev: 5'CTATTCTTTAGTTCC3'

Seq. P3. Primer forward Nef: 5'ATGGGTGGCAAGTGG3'

Seq. P4. Primer reverse Nef: 5'TCAGCAGTCCTTGTA3'

Seq. P5. Primer forward Gag: 5'ATGGGTGCGAGAGCG3'

Seq. P6. Primer reverse Gag: 5'TTATTGTGACGAGGG3'

Seq. P7. Primer forward IL-12: 5'ATGTGGCCCCCTGGG3'

Seq. P8. Primer reverse IL-12: 5'TTAGGAAGCATTCAG3'

Seq. P9. Primer forward IL-15: 5'ATGAGAATTTCGAAA3'

Seq. P10. Primer reverse IL-15: 5'TCAAGAAGTGTTGAT3'

Seq. P11.Primer forward Tat: 5'ATGGAGCCAGTAGAT3'

Seq. P12. Primer reverse Tat: 5'CTATTCCTTCGGGCC3'

Seq. P13. Primer forward Tat/Rev: 5'GGCCCGAAGGAAATGGCA GGAAGAAGC3'

Seq. P14. Primer forward Tat/Nef: 5' GGCCCGAAGGAAATGGGT GGCAAGTGG3'

Seq. P15. Primer forward Tat/Gag: 5' GGCCCGAAGGAAATGGGT GCGAGAGCG3'

Seq. P16. Primer forward Tat/IL-12: 5' GGCCCGAAGGAAATGTGGC CCCCTGGG3'

Seq. P17. Primer forward Tat/IL-15: 5' GGCCCGAAGGAAATGAGAAT TTCGAAA3'.

47. Process for preparing a vaccine according to the previous claims wherein the active principle, in its lyophilized form, is re-suspended in autologous serum for administration.

48. Use of Tat protein wild-type in its active form and/or its mutants and/or parts related to the protein or peptides or the DNA encoding for these proteins or

parts of them or peptides to make a protein or peptide or DNA vaccine, preventive and/or therapeutic, against AIDS, tumors, the syndromes and symptoms associated to HIV infection.

49. Use of Alum, ISCOM, RIBI, alone or in combination, to make a vaccine according to claim 1.

50. Use of paramagnetic beads coated with monoclonal antibodies anti-CD3 and anti-CD28 to make a vaccine according to claim 1.

Note 74: change the Anglo-Saxon notation into the corresponding italian one wherever in the text

Note 75: change the Figures into Tables 1 to 16.

Rome, 22 OCT 1998

The Representative

Dr. MARIA VITTORIA PRIMICERI

NOTARBARTOLO & GERVASI S.p.A.

MINISTRY OF INDUSTRY, COMMERCE AND HANDICRAFT

Italian Patent and Trademark Office ROME

****

AMENDMENT AND INSERTION REQUEST

Re: Patent Application for Industrial Invention No. RM97A000743 filed on 01.12.1997 in the name of ISTITUTO SUPERIORE DI SANITA' having title: : "HIV-1 or derivatives thereof Tat, alone or in combination for vaccination, prophylactic and therapeutical use against AIDS, tumours and related syndromes"

According to article 49 of D.P.R. dated June 22, 1979, No. 338, the undersigned Istituto Superiore di Sanità, having seat in Rome, Viale Regina Elena 299, through representatives Dr. Gemma GERVASI (Reg. No. 238) Dr. Diego Pallini (reg. No. 484), Dr. Giorgio Moretti (reg. No. 206), Dr. Angelo Passini (reg. No. 73), Ing. Giorgio Coggi (reg. No. 148), Dr. Livio Brighenti (reg. No. 475), D.ssa Maria Vittoria Primiceri (reg. No. 465), D.ssa Raffaella Consuelo Asensio (reg. No. 504), Dr. Giulio Mariani (reg. No. 329) (separately) of NOTARBARTOLO & GERVASI S.p.A., Via Savoia 82 - 00198 ROME, wherein has elected domicile in accordance with the law,

REQUESTS

this Office to make the amendments and insertions to the specification as hereinafter specified:

Note 1:

Page.	Line
26	18
29	6
30	13
31	4,21
51	18,19.20.21
55	6,7,12, and last line
56	1,10
59	4
63	6
64	16
68	6,7,9,10,11
70	2,4,5,6,7,8
71	after Table 12 lines: 2,3,4,5,6,7
76	2
77	after Table 16 lines: 1,5,6,7,8,9,10
81	2
82	15
85	2
87	21
90	after Table 20 lines: 2,3,4 and last but one and last line
91	after Table 22 lines: 2,3
92	last but one line
93	after Table 24 lines: 1,6
94	9,21
95	after Table 25 lines: 1,2
97	2,3,17
98	last but one line
103	after Table 32 line: 1
119	4

change the term «mg» into «µg» and the term «ml» into «µl».

Note 2: page 103, line 23: change «INFa, INFb» into «INFα, INFβ»

Note 3: page 137, line 23 and page 138, line 5: change «RGDD» into «RGDΔ».

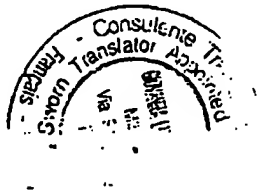
Note 4: page 44, line 1: change «CaOH» into «COOH».

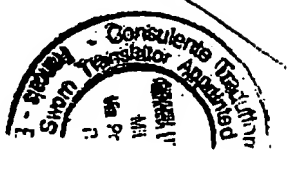
Rome, 30.11.1998

The Representative

Dr. MARIA VITTORIA PRIMICERI (signature)

NOTARBARTOLO & GERVASI S.p.A.


Consulente
Sworn Translator Appointed


Consulente
Sworn Translator Appointed

I, the undersigned, Giovanna Luisa SARALO
Sworn Translator, appointed by the Court of Milan, Italy. do hereby certify that the foregoing is the translation made by me of the documents in the Italian language which are attached hereto and that I conscientiously believe the said translation to be full, true and faithful, in witness whereof I have hereonto set my hand in Milan this 15th day of June 2005

Giovanna Luisa Saralo

Consulente Traduttore Tribunale Milano · Translator Appointed by Justice Court of Milano · Français - English - Deutsch · Sworn
GIOVANNA LUISA SARALO
MILANO
Via Pedgora. 6
ITALIA